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(54) Title: RECOMBINANT ENDOTOXIN-NEUTRALIZING PROTEINS

(57) Abstract

In general, the invention features a recombinant endotoxin-neutralizing polypeptide (RENP) characterized by (i) an amino acid sequence, (ii) an amino acid sequence and structure that facilitates selective and specific binding to lipopolysaccharide and (iii) once bound to the lipopolysaccharide, provides endotoxin-neutralizing activity. Preferably, the RENP is composed of an amino acid sequence similar to, but not identical to, an amino acid sequence of BPI, LBP, or both. preferably, the RENP contains an LPS-binding domain derived from the amino acid sequence of BPI, LBP or both. Preferably, the RENPs are covalently bound to a molecule which enhances the half-life of the polypeptide. The RENPs of the invention can be used in pharmaceutical compositions for therapeutic and prophylactic regimens, as well as in various *in vitro* and *in vivo* diagnostic methods.

RECOMBINANT ENDOTOXIN-NEUTRALIZING PROTEINS

Field of the Invention

5   This invention relates generally to the field of
recombinant, endotoxin-neutralizing proteins,
particularly to recombinant proteins which bind endotoxin
and block endotoxin-mediated activation of biological
systems.

Background of the Invention

10   Gram-negative infections are a major cause of
morbidity and mortality, especially in hospitalized and
immunocompromised patients. [Duma, *Am. J. of Med.*, 78
(Suppl. 6A):154-164 (1985); and Kreger et al., *Am. J.
Med.*, 68:344-355 (1980)]. Although available antibiotics
are generally effective in inhibiting growth of
Gram-negative bacteria, they do not neutralize the
pathophysiological effects associated with endotoxins.
Endotoxin is a heat stable bacterial toxin composed of
lipopolysaccharides (LPS) released from the outer
membrane of Gram-negative bacteria upon lysis
[Shenep et al., *J. Infect. Dis.*, 150(3):380-388 (1984)],
and is a potent stimulator of the inflammatory response.
Endotoxemia occurs when endotoxin enters the bloodstream
resulting in a dramatic systemic inflammatory response.
        Many detrimental *in vivo* effects of LPS result
from soluble mediators released by inflammatory cells.
[Morrison et al., *Am. J. Pathol.*, 93(2):527-617 (1978)].
Monocytes and neutrophils, which ingest and kill
microorganisms, play a key role in this process.
Monocytes and neutrophils respond to endotoxin *in vivo* by
releasing soluble proteins with microbicidal,

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proteolytic, opsonic, pyrogenic, complement-activating
and tissu -damaging effects. These factors m diate many
of the pathophysiological effects of endotoxin. For
example, tumor necrosis factor (TNF), a cytokine released
5 by endotoxin-stimulated monocytes, causes fever, shock,
and alterations in glucose metabolism and is a potent
stimulator of neutrophils. Other cytokines such as IL-1,
IL-6, and IL-8 also mediate many of the pathophysiologic
effects of LPS, as well as other pathways involving
10 endothelial cell activation by tissue factor, kininogen,
nitric oxide and complement.

        Endotoxin-associated disorders result from
extra-gastrointestinal exposure to LPS, e.g.
administration of LPS-contaminated fluids, or
15 Gram-negative infections. Endotoxin-associated disorders
can also result when the natural epithelial barrier is
injured and the normal Gram-negative flora breach this
barrier. For example, endotoxin-associated disorders can
occur (a) when there is ischemia of the gastrointestinal
20 tract (e.g., following hemorrhagic shock or during
certain surgical procedures), or (b) when systemic or
local inflammation causes increased permeability of the
gut to endotoxin or Gram-negative organisms. The
presence of endotoxin and the resulting inflammatory
25 response may result, for example, in endotoxemia,
systemic inflammatory response syndrome (SIRS), sepsis
syndrome, septic shock, disseminated intravascular
coagulation (DIC), adult respiratory distress syndrome
(ARDS), cardiac dysfunction, organ failure, liver failure
30 (hepatobiliary dysfunction), brain failure (CNS
dysfunction), renal failure, multi-organ failure and
shock.

        Examples of diseases which can be associated with
Gram-negative bacterial infections or endotoxemia includ
35 bacterial meningitis, neonatal sepsis, cystic fibrosis,

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inflammatory bow l dis ase and liv r cirrhosis,
Gram-negative pneumonia, Gram-n gative abdominal abscess,
hemorrhagic shock and disseminated intravascular
coagulation. Subjects who are leukopenic or neutropenic,
5 including subjects treated with chemotherapy or
immunocompromised subjects (for example with AIDS), are
particularly susceptible to bacterial infection and the
subsequent effects of endotoxin.

          Several therapeutic compounds have been developed
10 to inhibit the toxic effects of endotoxin, including
antibacterial LPS-binding agents and anti-LPS antibodies,
although each has met with limitations. For example,
Polymyxin B (PMB) is a basic polypeptide antibiotic which
binds to Lipid A, the most toxic and biologically active
15 component of endotoxin. PMB inhibits endotoxin-mediated
activation of neutrophil granule release *in vitro* and is
a potential therapeutic agent for Gram-negative
infections. However, because of its systemic toxicity,
this antibiotic has limited therapeutic use, and is
20 generally used topically. Combination therapy using
antibiotics and high doses of methylprednisolone sodium
succinate (MPSS) showed more promise as this regimen
prevented death in an experimental animal model of
Gram-negative sepsis. However, a clinical study using
25 MPSS with antibiotics in treatment of patients having
clinical signs of systemic sepsis showed that mortality
rates were not significantly different between the
treatment and placebo groups [Bone et al., *N. Engl. J.
Med.* 317:653 (1987)].

30   Antibodies that bind endotoxin have been used in
the treatment of endotoxemia. For example, hyperimmune
human antisera against *E. coli* J5 reduced mortality by
50% in pati nts with Gram-negative bacteremia and shock
[Ziegl r et al., *N. Engl. J. Med.* 307:1225 (1982)].
35 How v r, attempts to treat Gram-negativ sepsis by

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administration of anti-LPS monoclonal antibodies m t with
little or no success [Ziegler t al., *N. Engl. J. M d.*
324:429 (1991); Greenman et al., *JAMA* **266:1097** (1991);
Baumgartner et al., *N. Engl. J. Med.* **325:279** (1991)].

5   Another approach to treating endotoxemia involves
the use of cytokine blockers, such as IL-1 receptor
antagonists and anti-TNF antibodies, as well as the
soluble forms of the IL-1 and TNF receptors. However,
any given cytokine blocker blocks only the cytokine for
10   which it is specific, and fails to prevent the action of
other cytokines. Furthermore, blocking cytokines may
have other deleterious effects.

          Two soluble endotoxin-binding proteins,
lipopolysaccharide binding protein (LBP) and
15   bactericidal/permeability-increasing (BPI), play opposing
roles *in vivo* in the physiological response to endotoxin.
LBP is a soluble LPS receptor found in serum which binds
LPS with high affinity via interaction with the Lipid A
moiety [Tobias et al. (1986) J. Exp. Med. 164:777-793;
20   Tobias et al. (1989) J. Biol. Chem. 264:10867-10871].
LBP-LPS complexes stimulate monocyte activation through
interaction with the CD14 receptor on the surface of
monocytes, resulting in production of cytokines such as
TNF and IL-1 [Wright et al. (1989) J. Exp. Med.
25   170:1231-1241; Wright et al. (1990) Science 249:1431].
Thus, LBP acts as a transfer protein in LPS-mediated
stimulation of cytokine release. , Moreover, LBP increases
LPS activity in that a lower concentration of LPS is
required to stimulate monocytes in the presence of LBP
30   than in its absence.

          In direct contrast to LBP, BPI binds and
neutralizes endotoxin, preventing inflammatory cell
activati n. BPI, also known as CAP57 and BP
[Shafer et al., *Infect. Immun.* **45:29** (1984);
35   Hovde et al., *Infect. Immun.* **54:142** (1986)] is also

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bact ricidal by virtue of its interaction with th
Lipid A moi ty f LPS in the bacterial c ll wall. BPI
binds LPS, disrupts LPS structure and the cell wall, and
increases bacterial membrane permeability, resulting in
5 cell death [Weiss et al., J. Biol. Chem, 253:2664-2672
(1978); Weiss et al., Infection and Immunity 38:1149-1153
(1982)]. BPI retains its in vitro bactericidal activity
after protease cleavage, suggesting that BPI fragments
retain activity [Ooi et al., Clinical Research 33(2):567A
10 (1985)]. This observation was confirmed by Ooi et al.,
who showed that an N-terminal 25 kD fragment of BPI
exhibited both the in vitro bactericidal and permeability
increasing activities [Ooi et al., J. Biol. Chem.
262:14891 (1987)].

15 Molecular Structures of BPI and LBP
      The genes encoding BPI and LBP have been cloned
[Gray et al. (1989) J. Biol. Chem. 264:9505-9509;
Schumann et al., Science 249:1429-1431 (1990)]. BPI and
LBP are immunologically cross-reactive, contain a
20 hydrophobic leader sequence, and share significant amino
acid sequence homology over the entire length of the
molecules, with an overall amino acid sequence identity
of 44% [Tobias et al., J. Biol. Chem. 263:13479-13481
(1988); Schumann et al. supra]. BPI and LBP each
25 contains three cysteine residues. BPI contains two
glycosylation sites; LBP contains five potential
glycosylation sites.
      BPI is characterized by two distinct domains, an
N-terminal domain and a C-terminal domain, which are
30 separated by a proline-rich hinge region. The N-terminal
domain of BPI has strong LPS-neutralizing activity, while
the C-terminal domain of BPI has modest LPS-n utralizing
activity. LBP can also b divided into N- and C-terminal
domains, with th C-terminal domain being implicated in

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binding of LPS to macrophages and their subsequent
activation.

 The N- and C-terminal domains of BPI have a
striking charge asymmetry that is not shared by LBP. The
5 N-terminal domain of BPI, which is rich in positively
charged lysine residues, imparts a predicted pI > 10 to
the full-length molecule. In contrast, the C-terminal
domain of BPI is only slightly negatively charged. LBP,
which is a neutral protein, has no bactericidal activity
10 [Tobias et al., *J. Biol. Chem.* **263**:13479 (1988)]. This
suggests that the bactericidal activity of BPI results
from its overall cationicity.

 Table 1 provides a comparison of BPI and LBP
structure and function.

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Table 1

Comparison of BPI and LBP Structure and Function

	BPI	LBP
SYNTHESIS		
Site of synthesis	Neutrophil	Liver
Blood concentration	1-10 ng/ml	1-10 μg/ml
STRUCTURE		
Molecular mass	55 kD	60 kD
Glycosylation sites	2	5
Cysteine	3	3
EFFECTS ON LPS MEDIATED:		
neutrophil activation	Inhibits	Stimulates
monocyte activation	Inhibits	Stimulates
TNF release	Inhibits	Stimulates
IL-1 release	Inhibits	Stimulates
IL-6 release	Inhibits	Stimulates

* Four cysteines were reported by Schumann et al.
[*Science* 249:1429-1431 (1990)]. Subsequent DNA sequence
analysis by the present inventors determined that
Schumann's sequence was erroneous and that LBP contains
only three cysteine residues (see Figure 1).

Therapeutic intervention to block the inflammatory
effects of LPS would ameliorate the morbidity and
mortality associated with endotoxemia and septic shock.
Unfortunately, although BPI binds LPS with high affinity,
it has an extremely short half-life *in vivo*, thus
limiting its use in therapy. Native LBP has a longer
half-life but, upon binding of LPS, elicits a brisk
monocyte reaction which can facilitate release of
del t ri us quantities of cytokines.

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Early and specific diagnosis of
endotoxin-associated disorders is essential in th
identification of patients who have or who are at risk of
developing such disorders.

5   Precise identification of a site of Gram-negative
infection in a patient would assist the clinician in the
design and targeting of antibacterial therapy.

An ideal anti-endotoxin drug candidate and/or LPS
detection reagent would have a longer half-life and

10 effective, high-affinity endotoxin binding/inactivation
without monocyte stimulation. There is a clear need in
the field for specific diagnostic and therapeutic agents
which neutralizes the effects of endotoxin and has an
acceptably long half-life *in vivo*. The present invention

15 addresses these problems.

Summary of the Invention

Recombinant proteins are genetically engineered to
bind lipopolysaccharide (LPS) such that the endotoxin is
inactivated, thus preventing the endotoxin from inducing

20 the immunological cascade of events associated with
endotoxin-related disorders (e.g., activation of
monocytes, tumor necrosis factor (TNF) production).

In general, the invention features a recombinant
endotoxin-neutralizing polypeptide (RENP) characterized

25 by (i) an amino acid sequence, (ii) a sequence and
structure that facilitate specific binding to
lipopolysaccharide, (iii) provides endotoxin-neutralizing
activity upon LPS binding, and (iv) a half-life that is
enhanced relative to the half-life of BPI. Preferably,

30 the RENP is composed of an amino acid sequence similar
to, but not identical to, an amino acid sequence of BPI,
LBP, or both. Preferably, the RENP contains an
LPS-binding domain derived from th amino acid sequence
of BPI, LBP, or both. Pr ferred RENPs ar fusion

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proteins which bind LPS with the high affinity of BPI, but d n t c ntain the BPI amino acid sequences associated with BPI's short half-life.

Preferably, the RENPs are covalently bound to a
5 molecule which further enhances the half-life of the polypeptide. For example, the half-life enhancing molecule can be an immunoglobulin fragment, a half-life determining portion of LBP or LBP derivative, or polyethylene glycol. In related aspects, the invention
10 features DNA encoding an RENP of the invention, vectors and transformed cells containing DNA encoding an RENP, a method for producing RENPs, and detectably labeled RENPs.

A primary object of the invention is to provide an RENP which binds and inactivates endotoxin, and has a
15 half-life suitable for administration to a patient.

Another object of the invention is to provide a pharmaceutical composition containing a therapeutically effective amount of an RENP for use in treatment of endotoxin-related disorders.
20 Still another object of the invention is to provide endotoxin-neutralizing proteins for use in the detection of LPS. The RENPs can be bound to a label which can be detected or can be bound to a support for use in LPS-detection assays. LPS can be detected *in vivo*
25 to identify a site of infection in a subject or can be used in an *in vitro* assay to qualitatively or quantitatively detect LPS in a sample.

Another object of the invention is to provide endotoxin-neutralizing proteins that can be used to
30 produce endotoxin-free solutions and tools for use in, for example, various medical applications.

An advantage of the present invention is that the endotoxin-n utralizing proteins have a half-life in serum which is nhanc d r lativ to the half-life f
35 naturally- ccurring LPS-binding proteins, and bind LPS

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without triggering a significant, undesirable immune response.

Another advantage of the invention is that the RENPs can be administered to a patient immediately upon
5 identification of a symptom of an endotoxin-associated disorder.

Another advantage is that the endotoxin-neutralizing proteins can be administered prophylactically to a patient at risk of endotoxic shock
10 or other LPS-mediated condition.

An advantage of the invention is that various RENPs having binding specificity for LPS for detection of LPS either *in vivo* or *in vitro*.

Another advantage of the invention is that the
15 RENPs can be attached to a variety of detectable labels.

Yet another advantage of the invention is that the RENPs can be bound to a molecule which can interact with or which can be a portion of a solid support.

These and other objects, advantages and features
20 of the present invention will become apparent to those persons skilled in the art upon reading the details of the vectors, cell lines and methodology as more fully set forth below.

Brief Description of the Drawings

25   Figures 1A-1D are a comparison of the amino acid sequences of human LBP as described by Schumann et al. (LBPa) and as used herein (LBPb).

Figure 2 is a schematic diagram showing the various combinations of BPI, LBP, BPI variants, and/or
30 LBP variants which can be used to generate an RENPs of the invention.

Figures 3A-3D show the nucl otide and amino acid s qu nces of BPI.

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Figur s 4A-4C show the nucleotide and amino acid sequ nc s of LBP.

Figures 5A-5F are a comparison of the amino acid sequences of BPI and LBP from various species.

Figure 6 shows the amino acid sequence of $L_{1-197}B_{200-456}$ (NCY118).

Figure 7 is a graph showing the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) and $B_{1-199}L_{200-456}$ (NCY104) on biotinylated BPI binding to LPS.

Figure 8 is graph showing the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $B_{1-199}L_{200-456}$ (NCY104), or $B_{(S351->A)}$ (NCY105) protein on LPS activity in the chromogenic LAL assay.

Figure 9 is a graph showing FITC-LPS binding to monocytes in the presence of BPI or $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103).

Figure 10 is a graph showing the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) or $B_{1-199}L_{200-456}$ (NCY104), on TNF release by LPS in whole blood.

Figure 11 is a graph showing clearance of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) or $B_{1-199}L_{200-456}$ (NCY104) from mouse serum after intravenous injection.

Figure 12 is a graph comparing the efficacy of BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) in the protection to endotoxin challenge.

Figures 13A-13C are graphs showing the effects of BPI, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $L_{1-197}B_{200-456}$ (NCY118), $L_{1-198}B_{201-456}Fc$ (NCY144), $L_{1-59}B_{60-456}$ (NCY114), $L_{1-134}B_{135-456}$ (NCY115), $L_{1-359}B_{360-456}$ (NCY117), and B_{CAT9} (NCY139) on biotinylated BPI binding to LPS.

Figures 14A-14B are graphs showing the effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) and $B_{1-199}L_{200-456}$ (NCY104) on FITC-labeled LPS binding to human periph ral blood monocytes in the presence of 10%

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autologous s rum (14A) and in the absence of s rum and
presenc   f 0.5% human serum albumin (14B).

Figure 15 is a graph comparing the effects of LBP
vs. $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $B_{1-199}L_{200-456}$
5 (NCY104), $L_{1-359}B_{360-456}$ (NCY117) and PLL (poly-L-lysine)
on the stimulation of TNFα release by phorbol
ester-induced THP-1 cells in the absence of serum.

Figure 16 is a graph showing the effects of
various recombinant-endotoxin neutralizing proteins upon
10 LPS-mediated TNF production in THP-1 cells cultured
without serum.

Figures 17A-17H are graphs showing the clearance
of: BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103),
$B_{1-199}L_{200-456}$ (NCY104), and $L_{1-197}B_{200-456}$ (NCY118) (17A);
15 BPI, $L_{1-59}B_{60-456}$ (NCY114), $L_{1-134}B_{135-456}$ (NCY115), and B_{CAT9}
(NCY139) (17B); BPI, LBP, $L_{1-359}B_{360-456}$ (NCY117) and
$L_{1-197}B_{200-456}$ (NCY118) (17C); and BPI, LBP and
$L_{(1-198)}B_{(201-456)}Fc$ (NCY144) (assayed for both Fc and BPI)
in CD-1 mice (17D); LBP, $L_{1-275}B_{278-456}$ (NCY116),
20 $L_{1-359}B_{360-456}$ (NCY117), $L_{1-197}B_{200-456}$ (NCY118) (17E); LBP,
$L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103), $L_{1-134}B_{135-456}$
(NCY115), $L_{(1-198)}B_{(202-275)}L_{(274-456)}$ (NCY135), and
$L_{(1-134)}B_{(136-275)}L_{(274-456)}$ (NCY134) (17F); LBP (NCY102), L_{CAT6}
(NCY141), L_{CAT9} (NCY142), L_{CAT15} (NCY143) and BPI (17G);
25 and BPI, $L_{1-134}B_{135-456}$ (NCY115), and $L_{1-59}B_{60-456}$ (NCY114)
(17H).

Figure 18 is Western blot of BPI and $L_{1-197}B_{200-456}$
(NCY118) produced in *Pichia pastoris*.

Figure 19 is a graph showing the effects of BPI
30 and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) on endotoxin
activation of monocytes.

Figure 20 is a graph showing the protective
effects of $L_{1-197}B_{200-456}$ (NCY118) to endotoxin challenge
in mice.

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Description of the Preferred Embodiments

Bef re th present recombinant
endotoxin-neutralizing proteins, methods for providing
therapy to a patient suffering from an endotoxin-related
5   disorder, and compositions and method for diagnosis of a
condition associated with LPS are described, it is to be
understood that this invention is not limited to the
particular methodology, protocols, cell lines, vectors
and reagents described as such may, of course, vary. It
10 is also to be understood that the terminology used herein
is for the purpose of describing particular embodiments
only, and is not intended to limit the scope of the
present invention which will be limited only by the
appended claims.

15   It must be noted that as used herein and in the
appended claims, the singular forms "a", "and", and "the"
include plural referents unless the context clearly
dictates otherwise. Thus, for example, reference to "a
recombinant endotoxin-neutralizing protein" includes a
20 plurality of such proteins and reference to "the DNA
encoding the recombinant endotoxin-neutralizing protein"
includes reference to one or more transformation vectors
and equivalents thereof known to those skilled in the
art, and so forth.

25   Unless defined otherwise, all technical and
scientific terms used herein have the same meaning as
commonly understood to one of ordinary skill in the art
to which this invention belongs. Although any methods,
devices and materials similar or equivalent to those
30 described herein can be used in the practice or testing
of the invention, the preferred methods, devices and
materials are now described.

All publicati ns mentioned herein are incorporated
herein by reference for the purpose of describing and
35 disclosing the c ll lines, v ctors, and methodologies

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which are describ d in the publicati ns which might be
used in connecti n with the presently described
invention.

Definitions

5   By "lipopolysaccharide" or "LPS" is meant a
compound composed of a heteropolysaccharide (which
contains somatic O antigen) covalently bound to a
phospholipid moiety (lipid A). LPS is a major component
of the cell wall of Gram-negative bacteria.

10   By "endotoxin" is meant a heat-stable toxin
associated with the outer membranes of certain
Gram-negative bacteria, including the enterobacteria,
brucellae, neisseriae, and vibrios. Endotoxin, normally
released upon disruption of the bacterial cells, is

15 composed of lipopolysaccharide molecules (LPS) and any
associated proteins. The phospholipid moiety of LPS,
lipid A, is associated with LPS toxicity. When injected
in large quantities endotoxin produces hemorrhagic shock
and severe diarrhea; smaller amounts cause fever, altered

20 resistance to bacterial infection, leukopenia followed by
leukocytosis, and numerous other biologic effects.
Endotoxin is a type of "bacterial pyrogen," which is any
fever-raising bacterial product. The terms "endotoxin,"
"LPS," and "lipopolysaccharide" as used herein are

25 essentially synonymous.
         By "recombinant endotoxin-neutralizing
polypeptide", "RENP" or "recombinant LPS-neutralizing
polypeptide" is meant a protein which has been
genetically engineered and contains an LPS-binding

30 domain. Preferably, such recombinant LPS-binding
proteins bind endotoxin, have a relatively long half-life
in serum (e.g., compared to bactericidal/permeability
increasing (BPI) protein), and elicit no or relatively
little of the undesirable inflammatory side effects

associated with endotoxin and/or binding of LPS to
particular naturally occurring endot xin-binding prot ins
(e.g., lipopolysaccharide binding (LBP) protein).
"RENPs" of the invention do not occur naturally and are
5 distinct from those endotoxin-binding proteins that do
occur in nature, specifically BPI and LBP.

        By "LPS-binding domain" is meant an amino acid
sequence which confers specific and selective LPS binding
upon a polypeptide.
10   By "high affinity LPS binding" is meant an LPS
binding affinity greater than the LPS binding affinity of
LBP, preferably about the same or greater than the LPS
binding affinity of BPI.

        By "endotoxin-neutralizing activity" is meant a
15 biological activity associated with inhibition of the
toxic effects of lipopolysaccharide, e.g., by binding LPS
and preventing interaction of LPS with proteins and/or
receptors which mediate an undesirable immunological
response associated with endotoxin in a mammalian host.
20   By "recombinant" or "genetically engineered" is
meant a DNA sequence, or a polypeptide encoded thereby,
generated using nucleic acid manipulation techniques
(e.g., cloning, PCR, and/or fusion protein techniques).
"Recombinant" or "genetically engineered" DNA, and thus
25 the proteins encoded by such DNAs, do not occur in
nature.

        By "half-life" is meant the time required for a
living tissue, organ, or organism to eliminate one-half
of a substance introduced into it.
30   By "molecule which enhances the half-life" or
"half-life enhancing molecule" is meant chemical moiety
(e.g., bound via a chemical modification) which enhances
th biological half-life of a polypeptid with which it
is associated relative to the biological half-life of the
35 parent polypeptid . Chemical moieties includ an amino

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acid sequence or prot in. For xampl , wher a
p lyethylen glyc l (PEG) moi ty is c val ntly bound t a
protein so as to increase the half-life of the protein
relative to the un-PEGylated parent protein, the PEG
5   moiety is the "molecule which enhances the half-life" of
the protein.

By "half-life determining portion" of a
polypeptide is meant an amino acid sequence which is
associated with the biological half-life of the
10   polypeptide.

By "bactericidal/permeability increasing protein"
or "BPI" is meant a naturally occurring or recombinantly
expressed protein having the DNA and amino acid sequences
shown in Figures 3A-3D.
15   By "lipopolysaccharide binding protein" or "LBP"
is meant a naturally occurring or recombinantly expressed
protein having the DNA and amino acid sequences shown in
Figures 1A-1D and Figures 4A-4C.

By "BPI variant" is meant a protein having an
20   amino acid sequence similar to, but not identical to, the
amino acid sequence of BPI. "BPI variants" (a) bind LPS,
(b) competitively bind LPS in the presence of BPI or LBP,
and (c) inhibit the LPS-mediated production of TNFα by
human monocytes. In general, "BPI variants" contain the
25   amino acid sequence of BPI but with at least one of: 1)
an amino acid substitution; 2) an amino acid deletion; or
3) an amino acid addition, relative to the BPI amino acid
sequence.

By "LBP variant" is meant a protein having an
30   amino acid sequence similar to, but not identical to, the
amino acid sequence of LBP. "LBP variants" (a) bind LPS,
(b) competitively bind LPS in the presence of BPI or LBP,
and (c) inhibits production of TNFα by human monocytes.
In general, "LBP variants" contain the amino acid
35   s quenc of LBP but with at least one of: 1) an amino

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acid substitution; 2) an amino acid deleti n; or 3) an
amin acid additi n, relativ to the LPB amino acid
sequence.

By "detectable label" is meant any molecule
5 recognized in the art as a means for identifying and/or
detecting a protein to which the detectable label is
bound. Exemplary "detectable labels" include
radionucleotides, fluorescent moieties, biotin, and
antigenic molecules (e.g., a polypeptide which is
10 specifically bound by an anti-polypeptide antibody).
"Detectable labels" include a portion of a chimeric
protein where a portion of the chimeric protein can be
detected by, for example, binding of a detectably labeled
antibody or other detectably labeled molecule which
15 specifically binds the chimeric protein portion.

By "support" is meant a surface to which LPS or an
RENP of the invention can be bound and immobilized.
Exemplary supports include various biological polymers
and non-biological polymers.

20   By "condition associated with endotoxin",
"endotoxin associated disorder", or "endotoxin-related
disorder" is meant any condition associated with
extra-gastrointestinal (e.g., mucosal, blood-borne,
closed space) lipopolysaccharide, e.g., a condition
25 associated with bacteremia or introduction of
lipopolysaccharide into the blood stream or onto an
extra-gastrointestinal mucosal surface (e.g., the lung).
Such disorders include, but are not limited to,
endotoxin-related shock, endotoxin-related disseminated
30 intravascular coagulation, endotoxin-related anemia,
endotoxin-related thrombocytopenia, endotoxin-related
adult respiratory distress syndrome, endotoxin-related
renal failur , endotoxin-related liver disease r
h patitis, syst mic immune r sponse syndrom (SIRS)
35 resulting fr m Gram-negative infection, Gram-n gative

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neonatal sepsis, Gram-n gative m ningitis, Gram-negativ
pneumonia, neutropenia and/ r leucopenia resulting fr m
Gram-negative infection, hemodynamic shock and
endotoxin-related pyresis.

5   By "transformation" is meant a permanent genetic
change induced in a cell following incorporation of new
DNA (i.e., DNA exogenous to the cell). Where the cell is
a mammalian cell, the permanent genetic change is
generally achieved by introduction of the DNA into the
10 genome of the cell.

        By "transformed cell" is meant a cell into which
(or into an ancestor of which) has been introduced, by
means of recombinant DNA techniques, a DNA molecule
encoding a protein of interest.

15   By "promoter" is meant a minimal DNA sequence
sufficient to direct transcription. "Promoter" is also
meant to encompass those promoter elements sufficient for
promoter-dependent gene expression controllable for
cell-type specific, tissue-specific or inducible by
20 external signals or agents; such elements may be located
in the 5' or 3' regions of the native gene.

        By "operably linked" is meant that a DNA sequence
and a regulatory sequence(s) are connected in such a way
as to permit gene expression when the appropriate
25 molecules (e.g., transcriptional activator proteins) are
bound to the regulatory sequence(s).

        By "operatively inserted" is meant that the DNA of
interest introduced into the cell is positioned adjacent
a DNA sequence which directs transcription and
30 translation of the introduced DNA (i.e., facilitates the
production of, e.g., a polypeptide encoded by a DNA of
interest).

        By "mammalian subject" r "mammalian pati nt" is
meant any mammal for which the therapy of the invention

is desired, including human, bovine, equine, canine, and feline subjects.

The invention will now be described in further detail.

Nomenclature used to describe RENPs

In order to facilitate the discussion and description of the RENPs of the invention, each RENP is designated a specific formula to briefly describe the amino acid sequence of the protein, as well as the origin of specific portions of the protein. The portion of BPI in the recombinant protein is designated with the letter B, followed by an amino acid sequence numbering assignment corresponding to that shown in Figures 5A-5F for human BPI, wherein the mature N-terminus is designated as residue 1. The portion of LBP in certain LBP variants and chimeras is designated by the letter L, followed by an amino acid sequence numbering assignment corresponding to that shown in Figures 1A-1D for human LBP, wherein the mature N-terminus is designated as residue 1. To avoid confusion between the erroneous LBP amino acid sequence published by Schumann et al., *supra* (designated LBP-a) and the correct LBP amino acid sequence used in the RENPs of the invention (designated LBP-b) and presented in Figures 1A-1D. The differences between the DNA and amino acid sequences for "LBP-a" and "LBP-b" are presented in Table 2A below.

As an example of RENP nomenclature, $L_{1-197}B_{200-456}$ (NCY118) contains amino acid residues 1-199 of LBP fused at the C-terminus of the LBP portion to the N-terminus of amino acid residues 200-456 of BPI. $L_{1-197}B_{200-456}$, shown in Figure 6 has the N-terminal domain of LBP (having a putativ ndotoxin-binding domain) fused to the C-t rminal domain of BPI (having a putative LPS-clearing domain).

In this application, singl amino acid r sidue
substitutions are noted in parentheses, wher in the
original amino acid residue is indicated (using the
standard one letter code for amino acids), followed by
5 the substitute amino acid residue. For example, the BPI
variant having an alanine residue substituted for the
original serine residue at position 351 (which
substitution removes a glycosylation signal) is
designated $BPI_{(S351\rightarrow A)}$. In another example, in
10 $B_{(DS200\rightarrow DP)}$, a proline residue is substituted for the
serine residue at position 200. In this latter example,
the amino acid substitution produces a formic
acid-cleavable site.

As another example, the RENP LBP-BPI chimera
15 NCY103 is designated $L_{1-198(I43\rightarrow V)}B_{201-456(D206\rightarrow N)}$. In the
recombinant protein, the original isoleucine residue at
position 43 of the LBP portion is substituted with a
valine residue, and the original asparagine residue at
position 206 of the BPI portion is substituted with an
20 aspartate residue. The C-terminus of the LBP amino acid
sequence 1-198 having isoleucine substituted at position
43 is covalently bound to the N-terminus of the BPI amino
acid sequence 201-456 having valine substituted at
position 206.

25 The amino acid substitutions may be substitutions
wherein an original amino acid residue at a given
position is substituted with the residue at the
corresponding position in a different protein. $BPI_{(Xn\rightarrow Y)}$
is an example of such a substitution, wherein amino acid
30 residue X at position n in BPI is substituted with
residue Y which is found at position n in LBP (or rabbit
or bovine LBP). "X" and "Y" denote amino acid positions
in a primary amino acid sequence. "Y" as us d in this
cont xt is not to be confused with the symbol "Y"
35 d noting the amino acid residue tyrosine. $LBP_{(Xn\rightarrow Y)}$ is

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another example of such a substitution, wherein amino
acid residue X at p sition n in LBP is substitut d with
residue Y which is found at position n in BPI (or rabbit
or bovine BPI).

5   Amino acid residue insertion changes are noted in
parentheses, by indicating the amino acid residue after
which the insertion occurs, followed by the amino acid
residue after which the insertion occurs together with
the inserted residue or residues. For example,

10 $B_{(D200papain)}$ indicates that an amino acid sequence for
cleavage of the BPI variant by papain is inserted after
the aspartic acid at residue position 200.

TABLE 2A

Individual Sequence Differences Between
15   Schumann et al. and LBP as Used Herein

NUCLEIC ACID		PROTEIN	
Alpha	Beta	Alpha	Beta
A_{42}	C_{42}	$G_{129}YCL_{132}$	$V_{129}TAS_{132}$
C_{318}	T_{318}	S_{149}	F_{149}
G_{488}	(np)	A_{241}	$V_{241}MSLP_{245}$
(np)	C_{499}	L_{411}	F_{411}
T_{546}	C_{546}		
C_{548}	T_{548}		
(np)	$T_{824}CATGAGCCTTC_{835}$		
C_{1333}	T_{1333}		

(np) = not present in the sequence

          Table 2B describes some exemplary general classes
of RENPs of the invention. In the formulas in Table 2B,
n repr sents an amino acid residue position in the mature
30 sequ nce of BPI or LBP, x represents an amino acid
r sidu in a position which is C-terminal to n in th

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sequence of BPI or LBP, and y r pres nts an amino acid residue in a positi n which is C-terminal to x in the sequence of BPI or LBP. The symbols n, x and y denote the amino acid residue positions as they occur in the
5 mature sequence of the native protein, and not necessarily the positions as they occur in the variant protein.

Table 2B

10   ## Examples of RENPs

BPI variant (N-terminal frag.)	B_{1-n}
LBP variant (N-terminal frag.)	L_{1-n}
BPI variant (C-terminal frag.)	B_{n-456}
LBP variant (C-terminal frag.)	L_{n-456}
15 BPI variant (internal frag.)	B_{n-x}
LBP variant (internal frag.)	L_{n-x}
LBP-BPI chimera	$L_{n-x}B_{(x+1)-y}$
BPI-LBP chimera	$B_{n-x}L_{(x+1)-y}$
LBP-BPI chimera	$L_{n-x}B_{(x+1)-456}$
20 BPI-LBP chimera	$B_{n-x}L_{(x+1)-456}$
LBP-BPI chimera	$L_{1-n}B_{(n+1)-x}$
BPI-LBP chimera	$B_{1-n}L_{(n+1)-x}$
LBP-BPI chimera	$L_{1-n}B_{(n+1)-456}$
BPI-LBP chimera	$B_{1-n}L_{(n+1)-456}$
25 LBP-BPI-LBP chimera	$L_{1-n}B_{(n+1)-x}L_{(x+1)-456}$
BPI-LBP-BPI chimera	$B_{1-n}L_{(n+1)-x}B_{(x+1)-456}$

All of the constructs in Table 2B can also contain additional molecules which confer an enhanced half-life
30 upon the RENP (e.g., the RENP can be covalently bound to a polyethylene glycol moiety, or a portion of an immunoglobulin protein or other amino acid sequence which conf rs a half-life incr ased relative to th unmodified protein). The g neral sch me for generation of RENPs is
35 outlined in Figure 2.

<u>Production of RENPs</u>

        The RENPs of th inventi n minimally have
characteristics associated with (i) specific and high
affinity binding to lipopolysaccharide and

5 (ii) endotoxin-neutralizing activity. In general, the
amino acid sequence of RENPs is based upon an amino acid
sequence of BPI, LBP, or both. However, the amino acid
sequences of the RENPs are distinct from that of BPI and
LBP, i.e. the RENPs contain amino acid substitutions,

10 deletions, and/or additions relative to the amino acid
sequence of BPI or LBP. Thus, the RENPs of the invention
contain: 1) amino acid sequences of a
naturally-occurring LPS-binding protein (i.e., LBP and/or
BPI); and/or 2) amino acid sequences which do <u>not</u> occur

15 within a single naturally-occurring LPS-binding protein
(i.e., LBP or BPI). RENPs can thus be similar to, but
not identical to, LBP or BPI. For example, the RENPs can
be fragments of BPI and/or LBP, as the amino acid
sequences of such RENPs are similar to, but not identical

20 to, naturally occuring BPI or LBP. Moreover, the RENPs
of the invention generally have biological properties
distinct from and advantageous to either BPI or LBP.
RENPs of the invention include BPI variants, LBP
variants, and chimeric proteins composed of amino acid

25 sequences derived from BPI, LBP, BPI variants, and/or LBP
variants.

        For example, RENPs can contain an amino acid
sequence of BPI, where the BPI amino acid sequence 1) has
been altered at a site of glycosylation (e.g., insertion

30 or deletion of a glycosylation site); 2) contains a
neutral or anionic amino acid substituted at a cationic
residue of the BPI amino acid sequence (cationic
substitution variants); 3) contains an amino acid
substitution at a position normally occupied by cysteine

35 in the BPI s qu nce (cysteine substitution variants); 4)

contains an amino acid substitution wher th substituted
amino acid is the amino acid at th c rresponding
position in the LBP amino acid sequence; and/or
5) contains an insertion or deletion of one or more
5 secondary structure-altering amino acid residues.

        Exemplary BPI variants containing a glycosylation
site alteration include BPI variants having an amino acid
residue other than serine substituted for the serine
residue at position 351 of the BPI amino acid sequence.
10 BPI variants of this type are of the formula
BPI(S351->X), wherein X is any amino acid other than
serine. Preferably, the amino acid substituted at
position 351 is alanine. Other BPI variants having a
glycosylation site deleted can be generated by, for
15 example, other amino acid substitutions within the
glycosylation site.

        Additional exemplary BPI variants contain a
neutral or anionic amino acid substituted at a cationic
residue of the BPI amino acid sequence (cationic
20 substitution variants). For example, one or more of the
nonconserved positively-charged residues in BPI (i.e.,
those residues not found at the corresponding positions
in LBP) can be substituted with the corresponding residue
or residues in LBP, thus rendering BPI less cationic.
25 Preferably, the cationic substitution variant contains an
amino acid substitution in at least one of BPI amino acid
residue positions 27, 30, 33, 42, 44, 48, 59, 77, 86, 90,
96, 118, 127, 148, 150, 160, 161, 167, 169, 177, 185, or
198. The cationic substitution variant can contain
30 multiple amino acid substitutions. For example, the
cationic substitution variant can contain a neutral or
anionic residues at 1) BPI amino acid residue positions
27, 30, 33, 42, 44, 48, and 59; 2) BPI amino acid r sidue
positions 77, 86, 90, 96, 118, and 127; 3) BPI amino acid
35 residue positions 148, 150, 160, 161, 167, 169, 177, 185,

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and 198; or 4) BPI amino acid r sidue positions 27, 30,
33, 42, 44, 48, 59, 77, 86, 90, 96, 118, 127, 148, 150,
160, 161, 167, 169, 177, 185, and 198.

     Further example BPI variants contain an amino acid
5 substitution at a position normally occupied by cysteine
in the BPI sequence (cysteine mutant). The amino acid
selected for substitution at this site can be the amino
acid in the corresponding position in LBP. For example,
a cysteine residue in BPI (which is not conserved in LBP)
10 may be substituted with an alanine residue (the
corresponding residue in LBP). Preferably, the amino acid
substitution is at a cysteine residue at BPI amino acid
residue position 132, 135, or 175. Preferably, alanine
or serine is substituted for cysteine. More preferably,
15 alanine is substituted for the cysteine at position 132
of BPI. Cysteine substitution mutants of BPI can prevent
aggregation of the resulting RENPs during their
production or use.

     Another example of a BPI variant includes a BPI
20 variant having an amino acid substitution where the
substituted amino acid is the amino acid at the
corresponding position in LBP. The amino acid at the
corresponding position is determined by aligning the BPI
and LBP amino acid sequences so as to maintain the
25 highest level of amino acid sequence identity between the
two sequences. For example, an RENP having the formula
$B_{(Q329 \to S)}$ contains a substitution of the glutamine at BPI
residue position 329 with the serine residue at the
corresponding LBP residue position 327 (see Figures
30 5A-5F).

     Additional exemplary BPI variants contain an
insertion or deletion of one or more secondary
structure-alt ring amino acid residu s. For example, ne
or mor of the nonconserv d proline residues in BPI may

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be substituted with the corresponding non-prolin residue
in LBP.

Alternatively, or in addition to the amino acid
sequence of BPI and/or a BPI variant, the RENPs can
5   contain an amino acid sequence of LBP, where the LBP
amino acid sequence 1) has been altered at a site of
glycosylation (e.g., insertion or deletion of a
glycosylation site); 2) contains a cationic amino acid
substituted at a neutral or anionic amino acid of the LBP
10 amino acid sequence (cationic replacement mutant); 3)
contains an amino acid substitution where the substituted
amino acid is the amino acid at the corresponding
position in the BPI amino acid sequence; and/or 4)
contains an insertion or deletion of one or more
15 secondary structure-altering amino acid residues. The
LBP DNA and amino acid sequence used in the construction
of particular RENPs exemplified herein is the amino acid
sequence of human LBP in Figs. 5A-B.

Exemplary LBP variants contain a cationic amino
20 acid substituted at a neutral or anionic amino acid of
the LBP amino acid sequence (cationic replacement
variant). For example, one or more of the nonconserved
amino acid residues in LBP (at a position which
corresponds to a positively-charged residue in BPI) may
25 be substituted with the corresponding positively-charged
residue in BPI, and thus result in an LBP variant having
an increased positive charge, thus enhancing binding to
the negatively charged phosphate groups in LPS, and/or
facilitating interaction with the negatively charged
30 surfaces of Gram-negative bacteria. Positively-charged
residues include, by way of example, lysine, arginine,
and histidine. Preferably, the substituted cationic
amino acid is at least one of LBP amino acid residu
positions 77, 86, 96, 118, 126, 147, 148, 158, 159, 161,
35 165, 167, 175, 183, or 196. Cationic r placement

variants can contain multipl amino acid residu
substitutions at any c mbinati n of the amino acid
residues recited above.

Other exemplary LBP variants include an LBP
5 variant having an amino acid substitution where the
substituted amino acid is the amino acid at the
corresponding position in BPI. For example, $L_{(A401 \to D)}$
contains a substitution of the alanine residue of LBP at
position 401 with the aspartic acid residue at the
10 corresponding BPI residue position 403.

Further exemplary LBP variants contain an
insertion or deletion of one or more one or more
secondary structure-altering amino acid residues. For
example, one or more of the nonconserved amino acid
15 residues in LBP (at a position which corresponds to a
proline in BPI) may be substituted with a proline
residue. Preferably, such amino acid alterations alter
the secondary structure of the resulting LBP variant so
that it is more like the secondary structure of BPI.
20   Preferably, the RENPs of the invention contain at
least one LPS-binding domain of BPI, LBP, a BPI variant,
and/or a LBP variant. For example, the LPS-binding
domain can be derived from BPI and/or LBP amino acid
sequences 17-45, 65-99, and/or 141-167. Preferably, the
25 RENP has an LPS binding affinity that is greater than the
LPS binding affinity of LBP, more preferably an LPS
binding affinity that is the same or greater than the LPS
binding affinity of BPI. Preferably, the RENP has an LPS
binding affinity that is about 25-fold to 50-fold,
30 preferably about 50-fold to 100-fold, more preferably
about 100-fold to 300-fold greater than the LPS binding
affinity of LBP as determined by LPS binding or LPS
binding competition assays. The LPS binding affinity of
BPI is about 60-fold to 100-fold greater than the LPS
35 binding affinity of LBP.

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The RENPs can contain multiple LPS-binding domains derived from any f th s LPS-binding prot ins. For example, an RENP can be a multivalent chimeric protein (i.e., a fusion protein) composed of an LPS-binding

5 domain of BPI covalently bound (i.e., as in a fusion protein) to an LPS-binding domain of LBP. As used herein, a chimera means a protein comprising all or a portion of a first protein fused to all or a portion of a second protein, which resulting fusion protein may in

10 turn be fused to all or a portion of a third protein. Examples of chimeras include, by way of example, (a) a protein comprising a portion of LBP fused to a portion of BPI, (b) a protein comprising a portion of LBP fused to a portion of BPI which portion of BPI is in turn fused to a

15 portion of an immunoglobulin protein, or (c) a protein comprising a portion of LBP fused to a portion of BPI, which is in turn fused to a portion of LBP. Each protein portion of the chimera may comprise a fragment of the protein, a point mutant of the protein (i.e., a variant),

20 a deletion mutant of the protein, or a point and deletion mutant of the protein.

Examples of BPI fragments which can be incorporated into the RENPs of the invention include the BPI amino acid sequences 1-25, 1-85, 1-137, 1-135, 1-147,

25 1-159, 88-100, 148-161, 137-199, 44-159, 44-199, 135-199, 100-199, 162-199, 100-147. Examples of LBP fragments which can be incorporated into the RENPs of the invention include LBP amino acid sequences 1-43, 1-87, 26-135, 26-134, 86-99, 101-146, 101-197, 135-197, 137-197, 158-197,

30 160-197, and/or 147-159. The amino acid sequences of BPI and/or LBP can be comined in any order from N- to C-terminus to provide an RENP having sequences derived from BPI and/or LBP. For example, the RENPs can hav the sequenc s $B_{1-137}L_{137-197}$, $L_{1-43}B_{44-199}$, $B_{1-159}L_{158-197}$, B_{1-}

35 $_{135}L_{135-197}$, $L_{1-43}B_{44-159}L_{158-197}$, $B_{1-25}L_{26-135}B_{137-199}$, $B_{1-25}L_{26-}$

$134^B135-199$, $^L1-87^B88-100^L101-146^B148-161^L160-197$, $^B1-85^L86-99^B100-199$, $^B1-147^L147-159^B162-199$, $^B1-85^L86-99^B100-147^L147-159^B162-199$, $^L1-87^B88-100^L101-197$, or various combinations of other BPI and/or LBP fragments.

5   RENPs can share properties of both BPI and LBP. For example, fusing the N-terminal domain of LBP to the C-terminal domain of BPI results in an RENP which differs from LBP in that the chimera neutralizes endotoxin in whole blood and differs from BPI in that the chimera has

10 a longer circulating half-life *in vivo*. Such RENPs have significant diagnostic and therapeutic potential. As per the nomenclature described above, RENPs designated BPI-LBP contain all or a part of the N-terminal domain of BPI fused to all or a part of the C-terminal domain of

15 LBP. Likewise, RENPS designated LBP-BPI contain all or a part of the N-terminal domain of LBP fused to all or a part of the C-terminal domain of BPI.

         Where the RENP contains amino acid sequences derived from both BPI and LBP, the RENP is preferably

20 composed of a C-terminal fragment of BPI (or a BPI variant) and an N-terminal fragment of LBP (or an LBP variant). Preferably the C-terminal fragment of BPI (or a BPI variant) contains amino acid residues 60-456, 136-456, 199-456, 277-456, 300-456, 200-456, 136-361,

25 136-275, 200-275, or 200-361, more preferably 60-456, more preferably 199-359. The amino acid sequence of BPI from residue 199 to residue 359 contains a region required for neutralizing LPS, i.e., preventing LPS from stimulating an inflammatory response. Preferably, the N-

30 -terminal fragment of LBP (or an LBP variant) contains amino acid residues 1-59, 1-134, 1-164, 1-175, 1-274, 1-359, 1-134, or 1-197, more preferably 1-175. In addition to the specific amino acid sequences of BPI and LBP recited above, the RENP can also contain amino acid

35 residu s derived from the C-terminus of LBP (or an LBP

variant), pr ferably LBP (or LBP variant) amino acid
residu s 360-456 or 274-456.

Polypeptides which bind LPS can be identified
using any of several assays well known in the art such as
5   the 1) chromogenic LAL competition assay, 2) binding to
LPS immobilized on a surface, and 3) FITC-LPS assay for
binding to macrophages. The ability of a polypeptide to
neutralize endotoxin can also be determined using methods
well known in the art. Endotoxin neutralization assays
10 include assays to examine the ability of a polypeptide to
1) prevent LPS-induced TNF release in whole blood,
2) inhibit or prevent TNF production by THP-1 cells,
3) provide protection in a mouse endotoxin challenge
assay, and 4) reduce or prevent LPS-induced cytokine
15 release and/or mortality in an animal model. Each of
these assays are described in detail in the examples
section below. The results of the *in vitro* and *in vivo*
assays used herein are accepted in the art. The results
of these assays are predictive of relevant biological
20 activity *in vivo*, e.g. in humans.

Preferably, the RENPs of the invention have a
biological half-life (e.g., serum half-life) which is
enhanced relative to the biological half-life of BPI.
Preferabl, the half-life of the RENP is enhanced relative
25 to BPI such that the clearance time of the RENP is at
least 1.5-fold to 10-fold, preferably about 10-fold to
50-fold, more preferably about 50-fold to 100-fold, even
more preferably about 100-fold to 350-fold slower than
the clearance rate of BPI. The clearance rate values
30 representing these ranges are from about 8 ml/min to
1.5 ml/min, preferably 1.5 ml/min to 0.26 ml/min, more
preferably 0.26 ml/min to 0.13 ml/min, even more
pr ferably about 0.13 ml/min to 0.03 ml/min.

To enhanc the RENP half-life, the RENP can be
35 covalently bound to a mol cule which enhances the

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half-life of the polyp ptide. The half-life enhancing
mol cule can be any of a variety of half-life enhancing
molecules. Exemplary half-life enhancing molecules
include immunoglobulin fragments, a half-life determining
5 portion of LBP, a half-life determining portion of an LBP
variant, or polyethylene glycol (PEG), preferably a
half-life determining portion of LBP or an LBP variant.
Preferably, where the half-life enhancing molecule is a
portion of LBP or an LBP variant, the half-life enhancing
10 molecule is derived from the N-terminus of the LBP or LBP
variant amino acid sequence, more preferably from amino
acid residues 1-59, 1-134, 1-274, 1-359, 1-134, 1-164,
1-175, or 1-197, most preferably 1-164 or 1-175. Methods
of attachment of PEG moieties to a protein (i.e.,
15 PEGylation) are well known in the art and are exemplified
in U.S. patent nos. 4,179,337; 5,166,322; 5,206,344; and
PCT application serial no. PCT/US94/11624, published
April 28, 1995. °

     As used herein, an RENP-Ig chimeric protein is an
20 RENP which (i) contains a portion of BPI or LBP (at least
10 amino acid residues in length of (a) BPI, or (b) BPI
variant, or (c) LBP, and/or (d) LBP variant) fused at the
C-terminus to the N-terminus the Fc portion of an
immunoglobulin molecule, and (ii) is capable of (a)
25 binding to LPS, (b) competing with BPI or LBP for binding
to LPS, and (c) inhibiting the production of TNFα by
human monocytes. For example, the portion of the
immunoglobulin molecule is derived from an IgG molecule,
specifically from an IgG$_1$ heavy chain Fc domain. RENP-Ig
30 chimera is a fusion protein composed predominantly of
sequences derived from BPI, variant BPI, LBP and/or
variant LBP. The term "LBP-BPI-IgG chimera" indicates
that the RENP-Ig chim ra contains amino acid s quences
derived from both BPI (or a BPI variant) and LBP (or an
35 LBP variant).

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Id ntification of a half-life enhancing
polyp ptid sequence (.g., a p lypeptide deriv d from an
immunoglobulin, LBP, or LBP variant) can be accomplished
using methods well known in the art. For example, the
5 test polypeptide with and without the half-life enhancing
molecule bound to it are injected into an animal model to
determine the effects of the putative half-life enhancing
molecule. If the half-life of the polypeptide with the
molecule is enhanced relative to the half-life of the
10 polypeptide without the molecule, then the molecule is a
half-life enhancing molecule suitable for use in the
RENPs of the invention. For example, a putative
half-life enhancing amino acid sequence is incorporated
into a fusion protein with BPI. Both native BPI and the
15 BPI fusion protein are injected into mice. If the BPI
fusion protein has a half-life significantly greater than
the half-life of native BPI, then the amino acid sequence
in the BPI fusion has half-life enhancing
characteristics, and thus can be incorporated into the
20 RENPs of the invention.

Vectors and constructs

Any nucleic acid vector can be used to express DNA
encoding an RENP of the invention. The vectors
containing the DNA sequence (or the corresponding RNA
25 sequence) which may be used in accordance with the
invention may be any prokaryotic or eukaryotic expression
vector containing the DNA (e.g., cDNA) or the RNA
sequence of interest. A variety of suitable vectors are
publicly available and well known in the art. For
30 example, a plasmid can be cleaved to provide linear DNA
having ligatable termini. These termini are bound to
xogenous DNA having complementary, like ligatable
termini to pr vide a biologically functional recombinant

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DNA molecule having an intact r plicon and a desired
phenotypic prop rty.

A variety of techniques are available for DNA
recombination in which adjoining ends of separate DNA
5 fragments are tailored to facilitate ligation. The
vector is constructed using known techniques to obtain a
transformed cell capable of expression of the RENP. The
transformed cell is obtained by contacting a target cell
with a RNA- or DNA-containing formulation permitting
10 transfer and uptake of the RNA or DNA into the target
cell. Such formulations include, for example, plasmids,
viruses, liposomal formulations, or plasmids complexed
with polycationic substances such as poly-L-lysine or
DEAC-dextran, and targeting ligands. Transformed cells
15 containing a construct encoding an RENP of the invention
are also known in the art as "host vector systems".
Vectors for use in the construction of constructs
encoding the RENPs of the invention, as well as methods
for molecular cloning, nucleic acid manipulation, and
20 transformation of both eukaryotic and prokaryotic host
cells are well known in the art (see, for example,
Sambrook et al. <u>Molecular Cloning: A Laboratory Manual</u>,
2nd Ed., 1989, Cold Spring Harbor Laboratory Press, Cold
Spring Harbor, NY; hereby incorporated by reference with
25 respect to bacterial and eukaryotic vectors, and methods
and compositions for molecular cloning, nucleic acid
manipulation, and transformation techniques).

The constructs of the invention may include
promoter sequences to enhance expression of the
30 RENP-encoding DNA, as well as other sequences (e.g.,
enhancers) which facilitate or enhance DNA expression.
In addition, the RENP-encoding constructs can contain
th r c mponents such as a marker (.g., an antibiotic
resistanc g ne (such as an ampicillin resistance gene)
35 or β-galactosidase) to aid in selecti n of c lls

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containing and/ r xpressing the construct, an origin of
replicati n for stabl r plicati n f the construct in a
bacterial cell (preferably, a high copy number origin of
replication), a nuclear localization signal, or other
5 elements which facilitate production of the DNA
construct, the protein encoded thereby, or both.

In general, the RENPs of the invention are
constructed from a DNA sequence encoding BPI, a BPI
variant, LBP, an LBP variant, as well as various
10 half-life enhancing molecules known in the art such as
immunoglobulin fragments. Both BPI and LBP have been
cloned and their DNA and amino acid sequences determined
(Figures 3A-3B and 4A-4B, respectively). The DNA and
amino acid sequences of numerous immunoglobulins are
15 known in the art. For example, the DNA sequence of IgG,
IgG$_{2a}$, and IgG$_4$ are suitable for use to enhance the
half-life of the RENPs of the invention.

Expression of recombinant endotoxin-neutralizing
polypeptides

20   Techniques for obtaining expression of exogenous
DNA or RNA sequences in a host cell are known in the art
(see, for example, Sambrook et al., *supra*; hereby
incorporated by reference with respect to methods and
compositions for eukaryotic and prokaryotic expression of
25 a DNA encoding an RENP). Where the transformed cell is a
prokaryotic host cell, the preferred host is *Escherichia
coli*. Where the transformed cell is a eukaryotic host
cell, preferably the host cell is a mammalian cell or a
yeast cell. Preferably, the mammalian host cell is a
30 Chinese Hamster Ovary (CHO) cell. Preferably, the yeast
host cell is of the genus *Pichia*, more preferably a
strain of *Pichia pastoris*.

For pr karyotic expression, the construct should
contain at a minimum a bacterial origin of replication

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and a bacterial promoter op rably linked to th
RENP- ncoding DNA. F r eukaryotic expression, th
construct should contain at a minimum a eukaryotic
promoter operably linked to a DNA of interest, which is
5 in turn operably linked to a polyadenylation sequence.
The polyadenylation signal sequence may be selected from
any of a variety of polyadenylation signal sequences
known in the art. Preferably, the polyadenylation signal
sequence is the SV40 early polyadenylation signal
10 sequence. The eukaryotic construct may also include one
or more introns, which can increase levels of expression
of the DNA of interest, particularly where the DNA of
interest is a cDNA (e.g., contains no introns of the
naturally-occurring sequence). Any of a variety of
15 introns known in the art may be used. Preferably, the
intron is the human β-globin intron and inserted in the
construct at a position 5' to the DNA of interest.

Purification of RENPs

        Purification of the RENPs of the invention can be
20 performed according to any of a variety of protein
purification techniques known in the art including gel
electrophoresis, immunoprecipitation, ion exchange
chromatography, affinity chromatography, or combinations
thereof (see, for example, Guide to Protein Purification,
25 Deutscher, ed., Academic Press, Inc., San Diego, CA,
1990). Preferably, purification of RENPs is accomplished
by a combination of column chromatographic techniques.
For example, RENPs can be purified using a four-step
purification procedure using 1) a cation exchange column
30 (e.g., CM Sepharose), 2) an anion exchange column (e.g.,
Fast Q Sepharose), 3) a second cation exchange column
(e.g., CM Sepharose), and 4) a gel filtration sizing
column (e.g., Sepharos CL6B).

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Pharmaceutical compositions

The RENPs of the invention can be f rmulated as an active ingredient in a pharmaceutical composition. In general, the pharmaceutical composition contains a
5 therapeutically effective amount of an RENP and a pharmaceutically acceptable carrier. The pharmaceutical composition can contain one or more RENPs. The amount of RENP which constitutes a therapeutically effective amount will vary according to the time of administration (e.g.,
10 therapeutic or prophylactic administration), the disease or condition to be treated, the route of administration, and various patient-dependent factors such as age, weight, gender, and severity of disease. Specific therapeutically effective amounts appropriate for
15 administration are readily determined by one of ordinary skill in the art (see, for example, Remington's Pharmaceutical Sciences, 18th ed., Gennaro, ed., Mack Publishing Company, Easton, PA, 1990).

Pharmaceutically acceptable carriers suitable for
20 use in the RENP-containing pharmaceutical compositions of the invention are well known to those skilled in the art. Selection of the pharmaceutically acceptable carrier will depend upon a variety of factors including the RENP to be administered, the route of administration, and the
25 condition to be treated.

Pharmaceutically acceptable carriers suitable for use with the RENPs of the invention include, but are not limited to, 0.01-0.1 M and preferably 0.05 M succinate buffer or 0.8% saline. Additionally, such
30 pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Further, pharmaceutically acceptable carriers may include det rgents, phospholipids, fatty acids, or other lipid carriers. Exampl s of non-aqueous solv nts are propylene
35 glycol, polyethyl ne glycol, vegetable oils such as oliv

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il, and injectabl   rganic sters such as ethyl oleate.
Aqu ous carriers include water, alc holic/aqueous
solutions, emulsions or suspensions, including saline and
buffered media. Parenteral vehicles include sodium
5   chloride solution, Ringer's dextrose, dextrose and sodium
chloride, lactated Ringer's or fixed oils.

Pharmaceutically acceptable carriers for use with
the RENPs of the invention include lipid carriers. A
lipid carrier is any lipid-soluble substance which
10   inhibits protein precipitation and in which the proteins
of the subject invention are soluble. Lipid carriers can
be in the form of sterile solutions or gels, or can be
detergents or detergent-containing biological
surfactants. Examples of nonionic detergents include
15   polysorbate 80 (also known as TWEEN 80 or
polyoxyethylenesorbitan monooleate). Examples of ionic
detergents include, but are not limited to,
alykltrimethylammonium bromide. Exemplary lipid carriers
and methods for solubilizing BPI, and thus which can be
20   used in pharmaceutical compositions containing an RENP of
the invention, are described in USPN 5,234,912,
incorporated herein by reference.

Where the pharmaceutically acceptable carrier is a
lipid carrier, the lipid carrier may be a liposome. A
25   liposome is any phospholipid membrane-bound vesicle
capable of containing a desired substance, such as BPI or
BPI variant, in its hydrophilic interior. Intravenous
vehicles include fluid and nutrient replenishers,
electrolyte replenishers such as those based on Ringer's
30   dextrose, and the like. Preservatives, other
pharmaceutically active compounds, and other additives
may also be present, such as, for example,
antimicr bials, antioxidants, chelating agents, inert
gases and the lik .

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Disease conditions amenable to treatment with RENPs

Vari us diseas c nditions are amenable to
treatment using the recombinant LPS-neutralizing proteins
of the invention. In general, any condition of a
5 mammalian subject (e.g., human, canine, feline, or
bovine, preferably a human) which is associated with a
toxic effect of endotoxin can be treated by
administration of the RENPs of the invention.
Endotoxin-related disorders amenable to treatment
10 include, but are not limited to, endotoxin-related shock,
endotoxin-related disseminated intravascular coagulation,
endotoxin-related anemia, endotoxin-related
thrombocytopenia, endotoxin-related adult respiratory
distress syndrome, endotoxin-related renal failure,
15 endotoxin-related liver disease or hepatitis, systemic
immune response syndrome (SIRS) resulting from
Gram-negative infection, Gram-negative neonatal sepsis,
Gram-negative meningitis, Gram-negative pneumonia,
neutropenia and/or leucopenia resulting from
20 Gram-negative infection, hemodynamic shock and
endotoxin-related pyresis. Endotoxin-related pyresis is
associated with certain medical procedures, such as, for
example, trans-urethral resection of the prostate, and
gingival surgery. The presence of endotoxin may result
25 from infection at any site with a Gram-negative organism,
or conditions which may cause ischemia of the
gastrointestinal tract, such as hemorrhage, or surgical
procedures requiring extracorporal circulation. The
important role of endotoxin in hemorrhage (with
30 endogenous LPS translocation from the gut), trauma, and
sepsis is well known. One skilled in the art can
recognize additional conditions which can be treated
using the th rapy of the invention.

Th recombinant, endotoxin-neutralizing proteins
35 of the invention can also be administ red to a patient

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prophylactically, e.g. to a pati nt at risk of an
endot xin-related disorder. For xample, the RENPs can
be administered to a patient who has a Gram-negative
infection and is at risk of bacteremia, but who has not
5 yet exhibited symptoms associated with the toxic effects
of endotoxin. The RENPs can also be administered prior
to surgery where the risk of introduction of endotoxin
into the patient is substantial. One of ordinary skill
in the art can readily recognize other instances in which
10 prophylactic administration of a RENP is appropriate.
The conditions which identify an individual as being at
risk of an endotoxin-related disorder are well known in
the art.

Administration of RENPs

15   The recombinant, LPS-binding protein of the
invention may be administered using various methods well
known in the art. U.S. Pat. Nos. 5,171,739; 5,308,834;
and 5,334,584; each incorporated herein by reference,
describe methods and compositions for administration of
20 BPI, and thus can provide additional guidance for
administration of the RENPs of the invention. For
example, the recombinant, LPS-binding protein can be
administered by injection or inhalation. Administration
by injection can be an intravenous, intramuscular, or
25 subcutaneous route, or by direct injection directly into
a site of infection (e.g., tissue or body cavity).
Preferably, injection is intravenous. Administration by
inhalation is accomplished by delivery of the RENP to the
lungs via an aerosol delivery system or via direct
30 instillation. The aerosol may be nebulized. Various
devices and methods for aerosol drug delivery are well
known in th art. Methods for determining the
appropriate route of administration and dosage are
generally determined n a cas -by-case basis by the

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attending physician. Such d terminations ar routine to
on of rdinary skill in the art (se , for example,
Remington's Pharmaceutical Sciences, 18th ed., Gennaro,
ed., Mack Publishing Company, Easton, PA, 1990).

5   Therapeutically effective amounts of an RENP can
be determined according to methods well known to those
skilled in the art. Specific dosages will vary according
to a variety of factors, including the time of
administration (e.g., therapeutic or prophylactic
10   administration), the disease or condition to be treated,
the route of administration, the RENP to be administered,
and various patient-dependent factors such as age,
weight, gender, and severity of disease. The specific
dosage appropriate for administration is readily
15   determined by one of ordinary skill in the art according
to the factors discussed above (see, for example,
Remington's Pharmaceutical Sciences, 18th ed., Gennaro,
ed., Mack Publishing Company, Easton, PA, 1990). In
addition, the estimates for appropriate dosages in humans
20   may be extrapolated from determinations of the *in vitro*
LPS binding affinity of the RENP used, the amount of the
RENP effective to inhibit cytokine production by
mononuclear cells *in vitro*, the amount of RENP effective
to provide protection to LPS challenge, and/or various
25   other *in vitro* and *in vivo* assays indicative of the
biological activity of the RENP.

In general, the amount of RENP administered is an
amount effective to bind LPS and thereby inhibit the
undesirable biological activities associated with LPS
30   including monocyte and neutrophil activation, TNF
production, cytokine production, and other biological
phenomena triggered by LPS in endotoxin-related
disorders. Pr f rably, the amount of RENP administered
is an amount ff ctive to bind LBP and inhibit
35   LPS-mediated stimulation of neutrophils and mononuclear cells.

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In th rap utic administration of th RENPs of th
invention, an effective amount f an RENP is an amount
effective to bind to LPS and thereby inhibit LPS-mediated
stimulation of neutrophils and mononuclear cells in a
5 subject having an endotoxin-related disorder. As used
herein, "inhibit" means to inhibit at a level which is
statistically significant and dose dependent. The terms
"statistically significant" and "dose dependent" are well
known to those skilled in the art. In general, an
10 effective amount of an RENP in a pharmaceutical
composition for treatment of a patient having an
endotoxin-related disorder is an amount sufficient to
deliver to the subject a recombinant protein of the
subject invention at a concentration of between about
15 0.1 mg/kg of body weight and about 100 mg/kg of body
weight, preferably between about 1 mg/kg of body weight
and about 10 mg/kg of body weight. Preferably, the
RENP(s) is administered by injection, infusion, or as an
injected bolus so as to maintain a circulating RENP
20 concentration of about 1-10 μg/ml. The preferred
circulating RENP concentration can vary according to a
variety of factors, including the LPS binding affinity of
the specific RENP(s) administered.

As used herein, a prophylactically effective
25 amount of an RENP in a pharmaceutical composition for the
prevention of an endotoxin-related disorder is an amount
effective to bind LPS and prevent LPS-mediated biological
activity, e.g., LPS-mediated stimulation of monocytes and
neutrophils. In general, a prophylactically effective
30 amount of an RENP is an amount of a composition effective
to deliver between about 0.1 mg/kg of body weight and
about 100 mg/kg of body weight, preferably between about
1 mg/kg f body w ight and about 10 mg/kg of body w ight,
to th patient at risk of an endotoxin-related disorder.

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The invention also provides an article f
manufacture comprising packaging material and a
pharmaceutical composition contained within the packaging
material. The packaging material includes a label which
5 indicates that the pharmaceutical composition can be used
for treating a subject suffering from an
endotoxin-related disorder and\or for preventing an
endotoxin-related disorder (e.g., inflammation) in a
subject. The pharmaceutical composition contains a
10 therapeutically effective and/or prophylactically
effective amount of an RENP and a pharmaceutically
acceptable carrier.

Assessment of therapy
       The efficacy of the therapeutic or prophylactic
15 use of the RENPs of the invention can be determined by
monitoring patient symptoms associated with an
endotoxin-related disorder. Such symptoms, and methods
for monitoring, are well known in the art. For example,
where the RENP is used in the treatment of a patient
20 having an endotoxin-related disorder, the effectiveness
of the RENP therapy can be assessed by monitoring fever,
blood pressure, cytokine levels, and/or LPS levels in the
patient's blood stream. The presence of LPS in the blood
stream can be assayed as described above. Where the
25 patient is not responding, it may be desirable to
increase the dosage of the RENP pharmaceutical
composition or, where the patient is not responding
favorably, discontinue the RENP regimen.

Detectably-labeled RENPs
30   Various detectable labels, as well as methods of
attachment of such lab ls to a protein, are well known in
th art. Detectable labels can be any molecul
recognized in the art as a means for identifying and/or

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detecting a protein to which th detectable label is
bound. Exemplary "detectabl labels" include, but ar
not limited to radionucleotides, fluorescent moieties,
biotin, and antigenic molecules (e.g., a polypeptide
5 which can be specifically bound by an anti-polypeptide
antibody). Thus, detectable labels include a portion of
a chimeric protein (e.g., a fusion protein or genetically
engineered protein) where a portion of the chimeric
protein can be detected by, for example, binding of a
10 detectably labeled antibody or other detectably labeled
molecule which specifically binds the chimeric protein
portion. For example, where the RENP contains a portion
of the amino acid sequence of BPI, and an antibody which
specifically binds that amino acid sequence of BPI in the
15 context of the RENP is available, the BPI amino acid
sequence is the detectable label.

Methods for attaching (e.g., covalently binding) a
detectable label to a protein are well known in the art.
For example, methods for preparation of ^{125}I-labeled
20 proteins, biotin-labeled proteins, and FITC-labeled
proteins are well known. Methods for detectably labeling
antibodies are also well known in the art. Methods for
the production of antibodies for use in the subject
invention (e.g., anti-BPI, anti-LBP, anti-BPI variant,
25 anti-LBP variant, and anti-immunoglobulin fragment
antibodies) are well known in the art (see, for example,
Antibodies: A Laboratory Manual, Harlow and Lane, eds.,
Cold Spring Harbor Laboratory, Cold Spring Harbor, NY,
1988).

30 Detection of LPS in vitro

The detectably labeled RENPs of the invention can
be us d in various methods for th detection of LPS
either in vitro or in vivo. Samples for which in vitro
LPS detection is desirable include samples from a patient

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susp cted of having a Gram-negativ infecti n, and
samples from a pr duct for us in a medical application
(e.g., a recombinant protein solution where the protein
was expressed in *E. coli*). Patient samples include
5 samples of any body fluid, preferably blood or urine.
Samples may be pre-treated prior to testing by, for
example, concentrating the sample, or centrifugation to
remove cells and cellular debris.

In general, *in vitro* detection of LPS in a sample
10 suspected of containing LPS (test sample) is performed by
contacting the test sample with an RENP of the invention
for a time sufficient for the formation of RENP-LPS
complexes, and the RENP-LPS complexes detected. The
RENP-LPS complexes can be detected by virtue of a
15 detectable label attached to the RENP, or by the binding
of an anti-LPS antibody. Binding of the anti-LPS
antibody can subsequently be detected by virtue of a
detectable label bound to the antibody, or by the binding
of a detectably labeled anti-anti-LPS antibody to the
20 RENP-LPS-antibody complex.

The *in vitro* assay can be performed in solution by
mixing the sample with a solution containing RENP,
separation of RENP-LPS complexes (e.g., by
immunoprecipitation), and detection of the RENP-LPS
25 complexes formed, e.g., by virtue of a detectable label
bound to the RENP. Alternatively, the *in vitro* assay is
performed with RENP bound to a support, e.g., a polymeric
substrate such as a microtiter well or a latex bead.
Methods for binding proteins to a support are well known
30 in the art. For example, an anti-RENP antibody can be
bound to the support and the RENP subsequently bound to
the support via binding to the anti-RENP antibody. After
binding of the RENP to th support, the sample is th n
contacted with the support-bound RENP and any LPS in the
35 sample allowed to bind to the RENP. Unbound material is

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then washed away, and the RENP-LPS compl x s detected by
the binding f detectably labeled RENP r detectably
labeled anti-LPS antibody.

    The *in vitro* assay can also be performed as a
5 competition binding assay. For example, a sample
suspected of containing LPS (test sample) and a known
amount of detectably labeled RENP are incubated together
with a support having LPS bound to its surface. The test
sample and the RENP may be preincubated prior to contact
10 with the support-bound RENP. The level of detectably
labeled RENP bound to the support in the test sample is
compared to the level of detectably labeled RENP bound to
the support in a negative control sample (detectably
labeled RENP alone). A level of binding of detectably
15 labeled RENP in the test sample which is lower than
binding of detectably labeled RENP in the negative
control sample is indicative of the presence of LPS in
the sample.

    In an alternative embodiment, the competition
20 binding assay is performed with support-bound RENP. In
this latter assay, detectably labeled LPS (e.g.,
radiolabeled LPS) is mixed with the test sample suspected
of containing LPS, and the samples contacted with the
support-bound RENP, and the amount of detectably labeled
25 LPS bound to the support bound RENP detected. A level of
detectably labeled LPS bound to the support in the test
sample which is significantly lower than the amount of
detectably labeled LPS in the negative control sample
(radiolabeled LPS alone) is indicative of the presence of
30 LPS in the test sample.

    As is apparent from the description above, the
in vitro LPS assays of the invention can be performed
both qualitativ ly and quantitatively. For exampl ,
quantitative assays can be performed by comparing the
35 results btained with the test sample to results btain d

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with parallel sampl s containing known amounts of LPS.
Quantitative *in vitro* assays are indicative of, for
example, the severity of Gram-negative infection in a
patient sample from whom the sample was obtained, or a
5 degree of contamination where the test sample is a fluid
for administration to a patient (e.g., where the assay is
performed as a step in quality control). One of ordinary
skill in the art will appreciate upon reading the
above-described *in vitro* assays that numerous variations
10 of these assays can be performed without departing from
the spirit or the scope of the invention.

Detection of LPS *in vivo*

        Detectably labeled RENPs of the invention,
preferably RENPs having an increased LPS binding affinity
15 relative to LBP, can be used as a diagnostic to identify
a site of Gram-negative bacterial infection in a patient.
For example, a detectably labeled RENP is administered to
a patient suspected of having a Gram-negative infection.
Preferably, the detectable label is a radionucleotide
20 such as ^{123}I, ^{124}I, ^{125}I, ^{129}I, ^{131}I, or other beta-emitting
radionuclide which can be readily detected with either a
hand-held gamma radiation detection device or by nuclear
medicine scan. Alternatively, the detectable label is a
fluorescent molecule or other visually detectable label
25 which can be visualized during, for example, endoscopy.
Detection can be facilitated by increasing the ratio of
detectable label to RENP.

        The detectably labeled RENP is administered to the
patient in an amount sufficient for binding of the RENP
30 to the suspected infection site and detection of the
detectable label. The detectably labeled RENP can be
administer d by injection, preferably by ith r
intravenous injection or by direct inj ction into the
body cavity or tissue suspected of containing the

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infection sit . In g n ral, the amount f detectably
labeled RENP administer d will vary with according to
numerous variables including the RENP and detectable
label used, the location of the suspected site of
5   infection, the route of administration, and various
patient factors including size, weight, age, and
suspected severity of the disease.

     After administration, the detectably labeled RENP
is allowed to circulate to reach the site of infection
10 and/or incubate over the suspected site of infection.
Bound detectably labeled RENP is detected using methods
appropriate for the label used. For example, where the
detectable label is a radionucleotide, bound RENP is
detected using a radiation detecting device. Using this
15 method, the site and the extent of a Gram-negative
infection can be determined. Where desirable, the
detectably labeled RENPs can be used to label a site or
sites of infection which can then be imaged using any of
a variety of imaging techniques known in the art (e.g.,
20 X-ray, CAT scan, MRI, or PET scan).

LPS decontamination using RENPs
     The RENPs of the invention can also be used in the
decontamination of a product prior to its medical
application. For example, where a recombinant protein
25 has been produced by expression in *E. coli*, a solution
containing the recombinant protein can be applied to a
support having bound RENP (e.g., an affinity column).
LPS in the solution binds to the RENP bound to the
support, and the LPS-free solution is collected. If
30 necessary, the decontamination step can be repeated
multiple times until an acceptably low amount of LPS
(e.g. 0 to 0.001 ng/ml is detect d in th soluti n. Such
decontamination procedures using the RENPS of the

inventi n can be us d as a final st p in quality contr l of, for xampl , rec mbinantly produc d pharmaceuticals.

EXAMPLES

The following examples are put forth so as to
5 provide those of ordinary skill in the art with a complete disclosure and description of how to carry out the invention and is not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers
10 used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Centigrade, and pressure is at
15 or near atmospheric.

Example 1: Construction of RENPs

Specific examples of RENPs are described in Table 3, and are additionally designated by a construct name (e.g., NCY103) or lot number of the protein stock.

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Table 3

Examples of RENPs

	SEQUENCE	CONSTRUCT NAME OR LOT #	DESCRIPTION
5	BPI	NCY101	Native sequence
	$L_{1-197(143->V)}B_{200-456(N206->D)}$	NCY103	LBP-BPI chimera
	$B_{1-199}L_{200-456}$	NCY104	BPI-LBP chimera
	$B_{(S351->A)}$	NCY105	Glycosylation site deleted
10	$B_{(DS200->DP)}$	NCY106	Formic acid cleavage site inserted
	$L_{1-199}B_{200-456(S351->A)}$	NCY107	LBP-BPI chimera with glycosylation site deleted
	B_{1-199}	NCY108	N-terminal domain of BPI
	$B_{(1-190)}$	Lot #159699	N-terminal BPI fragment
	$B_{(1-236)}$	Lot #159695	N-terminal BPI fragment
15	$B_{(1-213)}$	Lot #159693	N-terminal BPI fragment
	$B_{1-199}Fc$	NCY110	N-terminal BPI-IgG chimera
	$B_{200-456}$	NCY112	C-terminal fragment of BPI
	$L_{1-59}B_{60-456}$	NCY114	LBP-BPI chimera
	$L_{1-134}B_{135-456}$	NCY115	LBP-BPI chimera
20	$L_{1-275}B_{278-456}$	NCY116	LBP-BPI chimera
	$L_{1-359}B_{360-456}$	NCY117	LBP-BPI chimera
	$L_{(1-164)}B_{(200-456)}$	Lot #164325	LBP-BPI chimera
	$L_{(1-175)}B_{(200-456)}$	Lot #164326	LBP-BPI chimera
	$L_{1-197}B_{200-456}$	NCY118	LBP-BPI chimera
25	$B_{(F61->C)}$	NCY119	Cysteine insertion
	$B_{(C132->A)}$	NCY120	Cystein substitution

SEQUENCE	CONSTRUCT NAME OR LOT #	DESCRIPTION
$B_{(C132->S)}$	NCY121	Cysteine substitution
$B_{(C135->S)}$	NCY122	Cysteine substitution
$B_{(C175->S)}$	NCY123	Cysteine substitution
Table 3 (continued)		
SEQUENCE	CONSTRUCT NAME OR LOT #	DESCRIPTION
$B_{(C132->A)(C135->S)(C175->S)}$	NCY124	Multiple cysteine substitution
$B_{(1-132->A)(C135->S)(C175->S)}$	NCY125	Multiple cysteine substitution
$L_{(1-134)}B_{(136-361)}L_{(360-456)}$	NCY133	LBP-BPI chimera
$L_{(1-134)}B_{(136-275)}L_{(274-456)}$	NCY134	LBP-BPI chimera
$L_{(1-198)}B_{(202-275)}L_{(274-456)}$	NCY135	LBP-BPI chimera
$L_{(1-198)}B_{(202-361)}L_{(360-456)}$	NCY136	LBP-BPI chimera
$B_{(1-41)}L_{(42-199)}B_{(200-456)}$	Lot #162303	BPI-LBP-BPI chimera
$B_{(1-190)(C173->A)}$	Lot #162305	N-terminal BPI fragment with cationic substitution
$B_{(K27->S)(K30->L)(K33->T)(K42->R)(K44->P)(K48->R)(R59->H)}$ (B_{CAT7})	NCY137	Cationic Substit. (7)
$B_{(K77->S)(K86->R)(K90->R)(R96->S)(K118->L)(K127->R)}$ (B_{CAT6})	NCY138	Cationic Substit. (6)
$B_{(K148->G)(K150->D)(K160->N)(K161->Q)(R167->Q)(K169->V)(K177->M)(K185->D)(K198->E)}$ (B_{CAT9})	NCY139	Cationic Substit. (9)
$B_{(K77->S)(K86->R)(K90>R)(K96->S)(K118->L)(K127->R)(K148->G)(K150->D)(K160->N)(K161->Q)(R167->Q)(K169->V)(K177->M)(K185->D)(K198->E)}$ (B_{CAT15})	NCY140	Cationic Substit. (15)
$L_{(S77->K)(R86->K)(R90->K)(S96->K)(L118->K)(R126->K)}$ (L_{CAT6})	NCY141	Cationic Repl. (6)

$L_{(G147->K)(D148->K)(N158->K)(Q159->K)(Q165->R)(V167->K)(M175->K)(D183->K)(E196->K)}$ (L_{CAT9})	NCY142	Cati nic Repl.(9)
$L_{(S77->K)(R86->K)(R90-K)(S96->K)(L118->K)(R126->K)(G147->K)(D148->K)(N158->K)(Q159->K)(Q165->R)(V167->K)(M175->K)(D183->K)(E196->K)}$ (L_{CAT15})	NCY143	Cationic Repl. (15)
$L_{(1-198)}B_{(201-456)}Fc$	NCY144	LBP-BPI-IgG chimera

Table 3 (continued)		
LBP	NCY102	native sequence
L_{1-199}	NCY109	N-terminal LBP fragment
$L_{1-199}Fc$	NCY111	LBP-Ig chimera
$L_{200-456}$	NCY113	C-terminal LBP fragment
$L_{(A132\rightarrow C)}$	NCY126	Cysteine insertion
$L_{(C81\rightarrow F)}$	NCY127	Cysteine substitution
$L_{(C81\rightarrow S)}$	NCY128	Cysteine substitution
$L_{(C135\rightarrow S)}$	NCY129	Cysteine substitution
$L_{(175\rightarrow S)}$	NCY130	Cysteine substitution
$L_{(C81\rightarrow F)(C135\rightarrow S)(C175\rightarrow S)}$	NCY131	Multiple cysteine substitution
$L_{(C81\rightarrow S)(C135\rightarrow S)(C175\rightarrow S)}$	NCY132	Multiple cysteine substitution

The proteins encoded by the LBP and $L_{1-359}B_{360-456}$ constructs facilitated the LPS-mediated cellular

15 response, indicating that LBP amino acid residues 275-359 are required for this LBP activity.

The cDNA sequences of BPI and LBP are shown in Figures 3A-3D and 4A-C, respectively, with each nucleotide designated numerically. DNA encoding the

20 RENPs can be prepared using a variety of techniques well known in the art, including protein fusion techniques, site-directed mutagenesis, and PCR (see, for example, Sambrook et al., *supra*; Zoller, M.J., et al., Methods Enzymol. 154:329 (1987)). For example, in the

25 construction of the RENP $L_{1-197}B_{200-456}$, the sequence "ATAGAT$_{723}$" and "ATTGAC$_{700}$" was chosen as a convenient site to insert a ClaI restriction site (ATCGAT) by which to r combine portions of both BPI (form r) and LBP (latt r). Oligonucleotide primers wer designed which

30 overlap this region but contain the ClaI s quence, and

w re synthesiz d on an ABI 380B synthesiz r (Applied
Bi systems Inc., F ster City, CA). Additional primers
were designed to bind to the 5' and 3'- ends of both
molecules, which primers contained NheI (5') and XhoI

5 (3') restriction sites for insertion into the vector.
These primers were used to amplify portions of the cDNA
molecules encoding amino acid residues 1-199 (A) and
200-456 (B) of LBP and BPI by cyclic DNA amplification.
The resulting DNA fragments were digested with the

10 appropriate restriction enzymes and then purified by gel
electrophoresis.

Example 2: Mammalian Expression

       In order to produce BPI, LBP, or RENPs of the
invention in mammalian cells, the cDNA sequences were

15 inserted into a suitable plasmid vector. A suitable
vector for such an application is pSE, which contains the
origin of replication and early and late promoters of
SV40, followed by multiple insert cloning sites, followed
by the termination sequences from the hepatitis B surface

20 antigen gene. An origin of bacterial DNA replication,
and the genes encoding ampicillin resistance and
dihydrofolate reductase were also included in the plasmid
for production of large amounts of DNA using bacterial
host cells. Similar vectors have been used to express

25 other foreign genes (Simonsen et al., *Biologicals* **22**:85
(1994). Another suitable vector, particularly for
rapidly obtaining small quantities of RENPs was pCIP4
(Invitrogen Corp., San Diego, California). pCEP4
contains a CMV promoter, followed by multiple insert

30 cloning sites, followed by SV40 termination sequences.
Also contained within the plasmid are an origin of
bacterial DNA replication, and th genes encoding
r sistanc to ampicillin and hygromycin B. With pCEP4
and pSE, the same insert cloning sites as pSE for easy

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insert shuttling betwe n the v ctors were used. Once
introduc d into mammalian cell hosts, this specialized
plasmid replicates as an episome, allowing semistable
amplification of introduced DNA sequences. The high gene
5 copy number is maintained through the selective pressure
of culture in the presence of hygromycin B.

A second expression system (EBV/293) was used to
rapidly obtain small quantities of recombinant proteins
of the subject invention when useful. This system was
10 constructed to use the same insert cloning sites as pSE
for easy insert shuttling, but utilized the Epstein-Barr
virus promoter (EBV) to drive heterologous expression
(pCEP4). Once introduced into mammalian cell hosts, this
specialized plasmid replicates as an episome, allowing
15 semistable amplification of introduced DNA sequences.
The high gene copy number is maintained through the
selective pressure of culture in the presence of
hygromycin plus G418. Similar expression systems are
commercially available (e.g., Invitrogen, Inc., San
20 Diego, CA).

Vector DNA was prepared for acceptance of BPI cDNA
by digestion with Nhe I and Xho I, and was subsequently
dephosphorylated by treatment with alkaline phosphatase.
The prepared fragments encoding BPI, LBP, or an RENP were
25 ligated into pSE or pCEP4, and the resulting recombinant
colonies were screened by agarose gel electrophoresis.
Subsequently, the DNA sequences were confirmed by
standard enzymatic sequencing methods (e.g., Sanger,
1974).

30   Expression plasmid DNA purified by either CsCl
gradients with Plasmid or Midi Kits (Qiagen, Chatsworth,
California) was used to transform Chinese hamster ovary
strain DUXB11 (pSE) and 293-EBNA c lls (Invitrogen Corp.,
San Di go, California) (pCEP4). Transfection was
35 p rformed using lipof ctin (Bethesda, Research Labs,

Gaith rsberg, MD) by standard methods. The resulting
transformed cells were sel ct d in GHT minus medium
(DUKXB11s) or in REM and 10% calf serum (293s). For the
DUKXB11s, clones were selected and were passed through

5 sequential rounds of culture in increasing concentrations
of methotrexate in order to amplify the DHFR gene and
associated heterologous genes. Supernatants from
transfected cells, either mixed populations or clones
derived from the mixed population, were assayed for RENPs

10 by ELISA using antibodies specific for BPI, LBP, or
immunoglobulin as appropriate.

Example 3:   Yeast Expression

BPI and $L_{1-197}B_{200-456}$ were successfully expressed
in the methylotrophic yeast *Pichia pastoris*. Pichia was

15 chosen as a suitable expression system for BPI and RENPs
due to its lack of LPS (endotoxin to which BPI and RENPs
bind) and its ability to produce high levels of mammalian
proteins.

Pichia pastoris strain GS115 (Invitrogen, San

20 Diego, California) was transformed with plasmids encoding
BPI and $L_{1-197}B_{200-456}$, and transformed colonies were
selected according to the procedures outlined by
Invitrogen (A Manual of Methods for Expression of
Recombinant Proteins in Pichia pastoris, Version 1.5,

25 Invitrogen, San Diego, California). For both BPI and
$L_{1-197}B_{200-456}$, protein was secreted into the medium in a
small-scale batch fermentation run. 116 ng/ml were
secreted for the one BPI construct assayed, and 14, 11,
and 10 ng/ml were secreted for the three constructs

30 $L_{1-197}B_{200-456}$ constructs assayed. Secretion was assayed
by enzyme-linked immunosorbant analysis (ELISA). The
majority of protein for both constructs was not s cr t d,
as shown by W stern blot analysis with a polyclonal
anti-BPI antib dy (INVN 1-2) (prepared by conventional

techniqu s by injecting rabbit with BPI) and alkalin
ph sphatase-c njugated goat anti-rabbit antibody. Th
Western blot is shown in Figure 18.

     Purified BPI from Chinese Hamster ovary cells
5 (CHOs) was used as a positive control (lane 12). In lane
1 a sample from untransformed GS115 cells served as a
negative control. The antibodies did not recognize any
proteins from the untransformed GS115 cells. The next
three lanes (2-4) were samples from colonies transformed
10 with the construct for BPI and the last 6 lanes (5-10)
were samples from colonies transformed with the construct
for $L_{1-197}B_{200-456}$. The amount of intracellular BPI or
$L_{1-197}B_{200-456}$ expressed in the batch fermentation run,
based on the amount of standard BPI loaded, was roughly
15 100 μg/ml of medium for the BPI and $L_{1-197}B_{200-456}$
colonies.

Example 4: Protein Purification

     BPI was purified from conditioned media using the
following four-step purification. BPI was captured on CM
20 Sepharose (Pharmacia LKB Biotechnology). The column was
washed in 50 mM Tris pH 7.4, and protein was eluted with
50mM Tris buffer pH 7.4 + 1 M NaCl. The eluate was
diluted 10X with 50mM Tris pH 8.5, run over Fast Q
Sepharose, and the flow-through was collected. BPI was
25 re-captured on CM Sepharose and again eluted as before.
Buffer exchange into 10 mM Succinate + 110 mM NaCl pH 6
was performed using Sepharose CL6B (Pharmacia LKB
Biotechnology). Finally, Tween 20 was added to the
formulated material to a final concentration of 0.05%.
30   LBP (NCY102) was captured from cell culture medium
on Fast S Sepharose (Pharmacia). The column was washed
with 50 mM Tris pH 7.4, and protein was elut d using 50mM
Tris pH 7.4 + 1 M NaCl. Th eluate was diluted 10X in
50mM Tris pH 8.5 and run over HiLoad Q Sepharose

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(Pharmacia). Protein was elut d with a 0-1 M NaCl
gradi nt in 50mM Tris pH 8.5. Appropriate fractions w re
pooled according to migration on SDS PAGE
electrophoresis. LBP concentration was diluted to 4.0
5 mg/ml, and the pH was adjusted to 7.0 with 100 mM HCl.

$L_{1-197(I43 \to V)}B_{200-456(N206 \to D)}$ was purified from cell
culture medium using the same method described for LBP.

$B_{1-199}L_{200-456}$ and $B_{(S351 \to A)}$ were purified using the
same protocol as for BPI, except that the size exclusion
10 step was omitted.

$L_{1-59}B_{60-456}$, $L_{1-134}B_{135-456}$ and B_{CAT6} were captured
on a Poros II HS cation exchange column (PerSeptive
Biosystems, Cambridge, MA) at pH 7.4. The column was
washed with 20 mM HEPES buffer at pH 7.5, and eluted with
15 20 mM HEPES pH 7.5 with 1 M NaCl. The eluate was diluted
5X in 20 mM HEPES pH 7.5 and applied to a Poros HQ anion
exchange column (PerSeptive) with the flow-through
applied directly to a POROS II HS column. The POROS II
HS column was eluted with 3.3 mM acetate, 3.3mM MES and
20 3.3 mM HEPES, pH 6.0 with a 0-1 M NaCl gradient.

$L_{1-359}B_{360-456}$ and $L_{(1-198)}B_{(201-456)}Fc$ were captured
from conditioned medium at pH 7.4 on a Poros II HS
column. The column was washed with 20mM HEPES buffer at
pH 7.5, and eluted with 20mM HEPES pH 7.5 + 1 M NaCl.
25 The eluate was diluted 10X with 20mM HEPES pH 7.5, loaded
on a second, smaller Poros II HS column, and eluted with
3.3 mM acetate, 3.3 mM MES and 3.3 mM HEPES, pH 6 with a
0-1 M NaCl gradient.

Example 5: BPI Activity Against N. meningitidis and N.
30 gonorrhoeae

BPI suppresses TNF release by human inflammatory
cells in respons to lipopolysaccharide (LPS) derived
from a wid rang of Gram-negative bacterial speci s. In
order to test the activity of BPI against Gram-negativ

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lipooligosaccharide (LOS) from th pathogenic bacteria
<u>Neisseria meningitidis</u> and <u>N. gonorrhoeae</u>, non-viable
bacteria were pre-treated with recombinant BPI and
incubated with human whole blood for 4 hours at 37°C.

5 Without BPI, <u>N. meningitidis</u> at 105 bacteria/ml
stimulated the release of 2.93 ± 0.53 ng/ml of TNF, while
<u>N. gonorrhoeae</u> was a more potent stimulator of TNF
release: 10_4 bacteria/ml induced 8.23 ± 0.32 ng/ml of
TNF. In both cases, 10 μg/ml BPI completely inhibited

10 TNF release. This indicates that BPI is able to bind and
detoxify LOS of these organisms, as well as bind LPS.
Thus, BPI can be useful as a therapeutic agent against
LOS-mediated tissue damage associated with these
pathogenic <u>Neisseria</u> species.

15 <u>Example 6:   biotinylated BPI Binding Competition Assays</u>
Competition assays for binding of LPS immobilized
on microtiter plates was performed using a modified
procedure described by Tobias et al., *J. Biol. Chem.*
264:10867 (1989). Briefly, Immulon 3 microtiter plates

20 (96-well, Dynatech Biotechnology Products, Chantilly, VA)
were coated with 1 or 4 μg of <u>S. minnesota</u> R595 Re LPS
(LIST Biological Labs, Inc., #304) in 50mM borate pH
9.5-9.8 + 20-25 mM EDTA overnight at 37°C. Blank,
non-LPS coated wells were included on each plate and

25 binding to these wells was used to determine non-specific
binding. Absorbance values from wells which were not
pre-coated with LPS consistently gave optical density
readings of less than 0.05. Plates were then washed
extensively under running distilled deionized water, then

30 dried at 37°C. Assay wells were blocked for 60 minutes
at 37°C with 1-2% very low endotoxin BSA (Sigma, St.
Louis, MO) prepared in pyrog n-fre Tris-buffer d saline
(50mM Tris pH 7.4 + 150 mM NaCl). The w lls w re
emptied, and biotinylated BPI was incubat d in the

presence r absenc of unlabeled BPI or recombinant
protein of the subj ct invention diluted in assay buffer
(pyrogen-free TBS + 1 mg/ml low endotoxin BSA, and 0.05%
Tween-20) was incubated in the LPS coated and uncoated

5   wells for 2-3 hours at 37°C in a total volume of 100
μl/well. After four washes in assay buffer, plates were
developed with streptavidin conjugated to alkaline
phosphatase (BioRad, Burlingame, California) followed by
100 μl of PNP substrate solution (Sigma) freshly prepared

10 from two 5 mg tablets dissolved in 10 ml substrate
buffer. Substrate buffer is prepared with 24.5 mg MgCl2,
48 ml diethanolamine, brought up to 400 ml, pH adjusted
to 9.8 and volume brought up to 500 ml. Absorbances were
read at 405 nm on a Vmax kinetic microplate reader

15 (Molecular Devices, Inc., Menlo Park, CA).

        The relative LPS binding affinities of BPI, LBP
and RENPs were tested in the competitive binding assay
described above using 10 ng/ml ^{biotinylated}BPI. In these
experiments, BPI inhibited ^{biotinylated}BPI binding to LPS

20 in a concentration-dependent manner (Figure 7). Modest
inhibition of ^{biotinylated}BPI-binding was observed using
NCY102 (LBP) and $L_{1-197(I43->V)}B_{200-456(N206->D)}$, suggesting
that BPI has either a higher affinity for LPS bound to a
surface or that LBP and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ bind

25 to a different site on LPS. $B_{1-199}L_{200-456}$, which contains
the N-terminal domain of BPI, competed with ^{biotinylated}BPI
at similar concentrations as unlabeled BPI, suggesting a
similar affinity and binding site.

        Competition between either $L_{1-197}B_{200-456}$ (NCY118)

30 or $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) with biotinylated
BPI occurred at similar concentrations, giving
overlapping curves (Figure 13A) indicating that the two
amino acid diff r nc s b tween th s two molecules
$[L_{1-197}B_{200-456}->L_{1-197(I43->V)}B_{200-456(N206->D)}$: (I43->V) and

35 (N206->D)] had no eff ct n affinity for immobilized LPS.

$L_{(1-198)}B_{(201-456)}$Fc (an IgG chimera consisting f
$L_{1-197}B_{200-456}$ linked t human IgG1 Fc constant region f
the immunoglobulin molecule) does not have an altered
ability to compete with biotinylated BPI (Figure 13A).

5 $L_{1-59}B_{60-456}$ and $L_{1-134}B_{135-456}$ showed a similar affinity for
LPS which affinity was very similar to that observed for
BPI, suggesting that the region between amino acid
residues 1-59 (or 1-134) probably plays a minimal role in
LPS binding (Figure 13B). Together with data showing the

10 $B_{1-199}L_{200-456}$ competes effectively with BPI (Figure 7),
these results indicate that amino acid residues 134-199
are important structural components of the high-affinity
LPS-binding domain of BPI.

The importance of the region between amino acid

15 residues 134 to 197 in LPS affinity was further
demonstrated by the markedly reduced affinity of B_{CAT9}, a
mutant in which all of the cationic amino acids of the
BPI molecule (particularly the cationic residues of BPI
amino acids 134-200) are replaced with the corresponding

20 amino acid residues found in LBP. These changes resulted
in a molecule with binding affinity for LPS which was
more similar to that of LBP than BPI (Figure 13C, and
Figure 7). Amino acid residues 360 to 456 of BPI are
apparently not involved in LPS binding as demonstrated by

25 the relative inability of $L_{1-359}B_{360-456}$ to displace
biotinylated BPI from LPS (Figure 13C). The apparent
binding affinity of $L_{1-359}B_{360-456}$ for LPS is similar to
that of LBP and B_{CAT9}, which affinity is approximately two
orders of magnitude lower than the apparent affinity of

30 BPI for LPS.

Thus, the domain of BPI which participates in
binding to immobilized LPS is localized in the N-terminal
half f the BPI mol cule, since $B_{1-199}L_{200-456}$ has the
greatest ability to displace nativ BPI from LPS coated

35 onto microtiter plates. This domain of BPI has b en more

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specifically localized to a region between amino acid residu s 134-199.

Example 7:   Chromogenic LAL Assay

To test the relative abilities of BPI, LBP and RENPs to neutralize LPS *in vitro*, these proteins were tested for inhibitory activity in the chromogenic LAL assay. Briefly, BPI and RENPs (25 μl of 0-200 μg/ml) were pre-incubated for 1 hour at 37°C with 1 EU/ml of *E. coli* 0111:B4 LPS, (Whitaker Biologicals, Walkersville, MD). The mixtures were then tested for LAL activity using the chromogenic LAL assay kit (Whitaker Biologicals, Walkersville, MD). The results are shown in Figure 8 and Table 4. LPS was neutralized by the various proteins tested in the order of:

$B_{(S351 \to A)} \geq BPI > L_{1-197(I43 \to V)}B_{200-456(N206 \to D)} > B_{1-199}L_{200-456} > LBP$. Several studies were carried out with different lots of each protein and the IC_{50} values were determined. The averaged IC_{50} values are shown in Table 4.

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Table 4

LPS Inhibition in the Chromogenic LAL Assay

Product	IC_{50} ($\mu g/ml$)	No. of test
$B_{(S351 \to A)}$	1.5	(n=1)
BPI	5.2 ± 3.3	(n=10)
$L_{1-197(I43 \to V)} B_{200-456(N206 \to D)}$	28.0 ± 20.0	(n=4)
$B_{1-199} L_{200-456}$	40.0	(n=1)
LBP	65.0 ± 31.0	(n=4)

These results demonstrate that BPI neutralizes LPS activity in the LAL assay at lower concentrations than LBP. $B_{1-199} L_{200-456}$, which contains the N-terminal domain of BPI, effectively competes with BPI for binding to LPS (see Figure 7) but is a relatively poor inhibitor of LPS in the LAL assay. These results indicate that the N-terminal (LPS-binding) domain of BPI alone does not account for the neutralizing activity of BPI in the LAL assay. $L_{1-197(I43 \to V)} B_{200-456(N206 \to D)}$ was a more potent inhibitor than LBP or $B_{1-199} L_{200-456}$, suggesting that the C-terminal domain of BPI plays a very important role in endotoxin neutralization in the LAL assay.

Additional results of LPS neutralizing activity in the chromogenic LAL assay are shown in Table 5. $L_{1-197(I43 \to V)} B_{200-456(N206 \to D)}$, $L_{1-59} B_{60-456}$, and $L_{1-134} B_{135-456}$ shar th C-terminal half of the BPI molecul , again indicating that this domain plays an important role in LPS-n utralizing activity. Also, these data indicat that th 199-456 region is most important in LPS

neutralization since adding BPI amino acid residues
between 136-456 or 60-456 did not improve LPS
neutralizing activity. Together with the LPS binding
data, these results further indicate that the C-terminal
5 half of BPI is important for neutralization, while the
N-terminal sequence is more critical for LPS binding.

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Table 5

LPS Inhibition in the Chromogenic LAL Assay

Protein		IC50	n
BPI	Cumulative	1.58 ± 1.58	94
	Lot# 149718	1.57 ± 1.01	54
	Lot# 149719	1.69 ± 0.35	7
	Lot# 149722	1.70 ± 0.28	2
	Lot# 149724	1.41 ± 0.45	45
	Lot# 155794	1.95 ± 0.92	2
LBP	Cumulative	55.92 ± 30.53	8
	Lot# 151281	34.33 ± 7.45	6
	Lot# 151204	77.50 ± 24.45	2
$L_{1-197}(I43->V)B_{200-456}(N206->D)$	Cumulative 22.86 ± 16.28		54
	Lot# 151235	25.50 ± 0.71	2
	Lot# 151242	36.50 ± 2.12	2
	Lot# 151274	3.46 ± 2.18	38
	Lot# 159616	8.83 ± 4.91	4
$B_{1-199}L_{200-456}$	Cumulative	24.19 ± 6.42	9
	Lot# 151246	12.50 ± 0.26	3
	Lot# 152658	10.70	1
	Lot# 155737	40.18 ± 34.48	4
B_{1-199}	Cumulative	5.52 ± 5.05	17
	Lot# 151285	1.12 ± 0.00	2
	Lot# 155709	9.73 ± 1.18	3
	Lot# 155779	2.13 ± 0.81	2
$L_{1-59}B_{60-456}$	Lot# 155754	3.64 ± 1.64	5
$L_{1-134}B_{135-456}$	Lot# 155756	5.02 ± 3.11	5
$L_{1-275}B_{278-456}$	Lot# 155791	14.00 ± 2.65	3
$L_{1-359}B_{360-456}$	Lot# 155733	>100	4

Protein		IC50	n
$L_{1-197}B_{200-456}$	Cumulativ	12.75 ± 3.54	12
	Lot# 155758	10.25 ± 30.9	8
	Lot# 159619	15.25 ± 5.91	4
B_{CAT6}	Lot# 155785	1.97 ± 0.06	3
B_{CAT9}	Lot# 155762	29.60 ± 23.23	5
B_{CAT15}	Lot# 155788	7.87 ± 2.80	3
$L_{(1-198)}B_{(202-275)}L_{(274-456)}$	Lot# 159649	>100	3
$L_{(1-198)}B_{(201-456)}Fc$	Lot# 155760	12.15 ± 6.00	4
L_{1-199}		9.2	1
B_{1-199}		10.1 ± 0.92	5
$L_{(1-134)}B_{(136-275)}L_{(274-456)}$	Lot# 159643	22.00 ± 15.25	4

_____

B_{CAT9}, which contains the entire BPI sequence except for nine cationic residues between positions 148 and 197 showed very poor LPS-neutralizing activity, suggesting that these residues are important in LPS-neutralizing activity. Similarly, this compound was relatively ineffective at competing with native BPI for binding to LPS. These cationic residues may permit correct structural conformation of the molecule, since both $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and B_{CAT9} contain the C-terminal domain of BPI, yet $L_{1-197(I43->V)}B_{200-456(N206->D)}$ has potent neutralizing activity while B_{CAT9} does not.

Example 8: Inhibition of FITC-labeled LPS binding to human monocytes

The relativ LPs-binding affiniti s of RENPs of the inv ntion wer inv stigated by xamining the abilities f th RENPs to inhibit LPS binding to human

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peripheral blood monocytes. Blood collected in acid
citrate dextr se-containing VACUTAINER™ tubes (Becton
Dickinson, Rutherford, NJ) was diluted 1:4 in Hank's
balanced salt solution (HBSS) minus calcium and magnesium
5 (Gibco BRL, Grand Island, MD). Mononuclear cells were
isolated using Ficol-Paque (Pharmacia Inc., Piscataway,
NJ). Cells were washed three times in HBSS, then brought
up to an appropriate volume of RPMI 1640 with glutamine
and antibiotics to give approximately 1×10^6 cells/ml.
10 To one ml aliquots of cells, FITC-LPS was added to a
final concentration of 500 ng/ml. Tubes were closed and
incubated at 37°C on a rocking platform. At the end of
the incubation, cells were washed twice with PBS with
0.05% Human Serum Albumin and 0.02% sodium azide. FACS
15 analysis of the cells was performed on a FACStar flow
cytometer, Immunocytometry System, Becton Dickinson
(Mountain View, CA). The monocyte portion of the cell
population was determined by side scatter versus forward
scatter gating and confirmed by staining a separate
20 aliquot of cells with phycoerythrin-conjugated anti-DR
antibody (Becton Dickinson Immunocytometry Systems,
Milpitas, CA). Results are reported as logarithmic scale
mean fluorescence intensity.

       To determine the relative abilities of BPI or
25 $L_{1-197(I43->V)}B_{200-456(N206->D)}$ to inhibit LPS binding to
human peripheral blood monocytes, isolated human
peripheral blood mononuclear cells were incubated with
10% human serum containing 500 ng/ml FITC-conjugated _E._
coli 055:B5 LPS in the presence or absence of BPI or
30 $L_{1-197(I43->V)}B_{200-456(N206->D)}$. Binding of FITC-LPS to
monocytes could be inhibited by increasing concentrations
of both BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (Figure 9).
Thus $L_{1-197(I43->V)}B_{200-456(N206->D)}$ has BPI-like activity,
d spit the fact that $L_{1-197(I43->V)}B_{200-456(N206->D)}$ c ntains
35 th N-terminal domain f LBP. These data, al ng with th

results f the LPS n utralizati n studies sh wn in Figur
8, suggest that the C-terminal d mains of BPI and LBP,
and not the N-terminal domains, determine whether the
proteins inhibit or mediate LPS activation of cells.

5   Further studies were undertaken to determine the
effects of BPI, LBP, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and
$B_{1-199}L_{200-456}$ on FITC-labeled LPS binding to peripheral
blood monocytes in the presence and absence of serum. In
a serum-free FITC-labeled LPS binding system where no LBP

10 is available, FITC-labeled LPS does not bind to cells.
In contrast recombinant LBP facilitated LPS binding to
cells at concentrations as low as 100 ng/ml.
$B_{1-199}L_{200-456}$ also facilitated binding, although to a
lesser extent. Neither BPI or $L_{1-197(I43->V)}B_{200-456(N206->D)}$

15 promoted significant binding of LPS to cells. These data
indicate that the C-terminal domain of LBP is active in
LPS binding to cells. The N-terminal domain of BPI may
exert an inhibitory influence on LPS binding to cells
mediated by the C-terminal domain of LBP because

20 $B_{1-199}L_{200-456}$ was less active than LBP.

          Normal human serum contains about 1-10 μg/ml LBP.
In the presence of 10% autologous serum, BPI and
$L_{1-197(I43->V)}B_{200-456(N206->D)}$ potently inhibited FITC LPS
binding to monocytes, with BPI showing slightly greater

25 potency. $B_{1-199}L_{200-456}$ had marginal activity, and LBP had
no effect (Figure 14A). These data indicate the
importance of the BPI C-terminus in this test of LPS
neutralization. $B_{1-199}L_{200-456}$, which lacks the C-terminal
domain of BPI, is approximately two orders of magnitude

30 less potent at blocking LPS binding. LBP, as expected,
had no effect. Thus, BPI can intervene in the sepsis
cascade by preventing LPS from binding to monocytes and
causing releas of TNFalpha.

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Example 9: THP-1 Cell TNF Production Assay

THP-1 cells w re obtained fr m the American Tissu
Culture Collection (Rockville, MD) and were maintained in
REM medium containing 10% fetal bovine serum, 2 mM
5 L-glutamine, 100 units penicillin and 100 μg/ml
streptomycin. Cells were passed at 2 x 10^5 cells/ml
every 3 days. Responsiveness of THP-1 cells to LPS was
induced by culturing the cells for 48 hours in REM medium
containing 10% fetal calf serum, 2 mM L-glutamine, 100
10 units penicillin, 100 μg/ml of streptomycin and
100 nM PMA at 37°C in a humidified atmosphere with
5% CO_2. Cells were cultured in 96-well flat-bottomed
tissue culture plates at 1-2 x 10^5 cells per well in a
final volume of 200 μl. After 48 hours, adherent cells
15 were washed three times with 200 μl of medium without
serum. To 180 μl of medium without serum but with 0.5%
HSA, LPS (10 μl at 200 ng/ml) and/or BPI, LBP or other
RENPs were added (10 μl at 0-2 mg/ml) and the cells were
cultured for an additional 4 hours. After 4 hours,
20 supernatants were transferred to a U-bottomed 96 well
plate and the plate was centrifuged (500 x g, 12 min) to
pellet any cell debris. Supernatants were then stored in
a second plate at -20°C until assayed for TNF by ELISA.
To further identify the regions of BPI which
25 contribute to LPS-neutralizing activity, and the domains
of LBP which are responsible for transducing the LPS
signal to cells, the abilities of RENPs to replace LBP
were compared under serum-free conditions. In these
experiments, cells of the promonocytic cell line THP-1
30 were induced to respond to LPS by culturing for 48 hours
with phorbol ester. After induction, cells were
stimulated with 19 ng/ml of LPS in the presence or
abs nce of the recombinant protein. In this system, TNF
r lease requir s a sourc of LBP. Data from these
35 exp riments (Figur 15) show that only LBP and

$L_{1-359}B_{360-456}$ stimulat d TNF r l as . Thus the domain f
LBP responsible f r facilitating LPS-induced TNF rel ase
is within amino acid residues 199-359. Interestingly,
$B_{1-199}L_{200-456}$ did not mediate TNF release in a serum-free
5 system. This may indicate that the N-terminal domain of
BPI binds too tightly to LPS to allow transfer of LPS to
CD14 on the macrophage surface. Figure 16 shows an
additional comparison of TNF production. Because
$L_{(1-198)}B_{(202-275)}L_{(274-456)}$ includes LBP domain 274-456 and
10 has activity, the active domain may comprise only
residues 274-359.

Example 10: LPS-Induced TNF Release In Whole Blood
        Peripheral blood from normal human volunteers was
collected in heparin-containing VACUTAINER™ tubes (Becton
15 Dickinson). To one milliliter of whole blood, BPI, a
protein of the subject invention, or buffer control was
added, followed by 1 ng/ml E. coli 055:B5 refined
standard endotoxin (RSE) (Whitaker Bioproducts). Samples
were incubated in closed microtubes at 37°C for 4 hours
20 on a rocking platform. At the end of the incubation,
samples were centrifuged for 5 minutes at 500 x g at 4°C,
the plasma collected and frozen on dry ice until assayed
for the presence of cytokines. TNF levels were
determined by ELISA using human recombinant TNF
25 (Genentech Inc., South San Francisco, CA) as a standard.
        In later studies it was determined that BPI
activity in whole blood is inhibited by heparin, and the
anticoagulant was changed to citrate. In these
experiments, to 120 μl of citrated whole blood, 20 μl of
30 BPI or an RENP (at 0-1 mg/ml) or buffer control, 20 μl of
100 ng/ml of E. coli 055:B5 LPS was added to stimulate
cells in whole blood samples. Th se experiments w re
performed in polypropyl ne microtiter plates (Costar,
Cambridge, MA). After the 37°C incubation step, the

plat s w re centrifug d 15 min at 500 x g at 4°C and the
plasma removed f r assaying.

　　　　To test the effects of BPI, LBP, and RENPs on LPS
activation of TNF production in whole blood, BPI, LBP,

5　$L_{1-197(I43->V)}B_{200-456(N206->D)}$, or $B_{1-199}L_{200-456}$ was mixed
with heparinized blood, and LPS was added to the
resulting mixture. The blood was incubated for four
hours at 37°C, and TNF in the plasma was measured as
described, supra. Results are shown in Figure 10.

10　$L_{1-197(I43->V)}B_{200-456(N206->D)}$ was the most potent at
blocking TNF release, followed by BPI as the next most
potent blocker. $B_{1-199}L_{200-456}$ and LBP had essentially no
effect. Thus, in whole blood, $L_{1-197(I43->V)}B_{200-456(N206->D)}$
proved to be the most potent inhibitor of LPS-mediated

15　cytokine stimulation.

　　　　When experiments were performed in citrated rather
than heparinized whole blood, endotoxin-neutralizing
activity of BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ were
equivalent (Table 6). In experiments in which

20　recombinant proteins were preincubated with endotoxin
before addition to whole blood, the activities of these
compounds fell roughly into two groups. BPI,
$L_{1-197(I43->V)}B_{200-456(N206->D)}$, B_{1-199}, B_{CAT6}, B_{CAT15},
$L_{1-59}B_{60-456}$, $L_{1-134}B_{135-456}$, and $L_{1-197}B_{200-456}$ possess

25　LPS-neutralizing activity, while LBP, $B_{1-199}L_{200-456}$,
L_{1-199}, $L_{1-359}B_{360-456}$ and B_{CAT9} were relatively inactive.
Results with $L_{1-275}B_{278-456}$, B_{CAT9}, and $L_{(1-198)}B_{(201-456)}Fc$
were equivocal. When compounds were added to the blood
samples immediately prior to LPS, the IC50 values were

30　higher, but the same group of proteins showed activity.
These data further indicate the role of the C-terminal
region of BPI, demarcated by amino acid residues 200-359,
in LPS neutralization in a physiological nvironm nt such
as whol blood. Because L_{1-199} is not a pot nt

35　endotoxin-neutralizing prot in (see Tabl s 9 and 11), it

can b concluded that th C-t rminal domain of BPI must
significantly c ntribute t the endotoxin-neutralizing
activity of $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and $L_{1-197}B_{200-456}$.
All compounds which contain this sequence (201-359) are
5   active except B_{CAT9}, which was also inactive in other
assays possibly because the cationic amino acid residues
which were replaced may be important in configuring the
molecule. These data indicate that

$L_{1-197(I43->V)}B_{200-456(N206->D)}$ is equivalent to $L_{1-197}B_{200-456}$
10 in activity, thus implying that the amino acid
differences between these two proteins have no affect
upon LPS binding affinity.

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Table 6

LPS Inhibition in Human Whole Blood

Protein Pre-incubated	IC50 (µg/ml)	n	Not Pre-incubated	IC50 (µg/ml)	n
$L_{1-134}B_{135-456}$	0.15 ± 0.12	3	BPI	2.60 ± 1.52	5
$L_{1-197}B_{200-456}$	2.90 ± 3.59	12	$L_{1-134}B_{135-456}$	3.7 ± 1.60	2
$L_{1-59}B_{60-456}$	0.28 ± 0.25	3	$L_{1-199\backslash 7(I43->V)}B_{200-456}$ (N206->D)	7.13 ± 5.92	4
$L_{1-197(I43->V)}B_{200-456}$(N206->D)	0.16 ± 0.11	17	$L_{1-59}B_{60-456}$	15 ± 18.58	2
BPI	0.43 ± 0.49	13	$L_{1-197}B_{200-456}$	26.5 ± 0.71	2
$L_{(1-198)}B_{(201-456)}Fc$	18.00 ± 27.73	3	$L_{1-359}B_{360-456}$	>100	1
$B_{1-199}L_{200-456}$	>100	3	B_{CAT9}	>100	2
$L_{1-359}B_{360-456}$	>100	3	$L_{(1-198)}B_{(201-456)}Fc$	>100	2
B_{CAT9}	11.50 ± 3.54	2*	$B_{1-199}L_{200-456}$	ND	
B_{1-199}	0.73 ± 0.48	6	B_{1-199}	4.0	1
L_{1-199}	>100	2	L_{1-199}	>100	1
B_{CAT15}	0.21 ± 0.26	3			
B_{CAT8}	0.27 ± 0.25	2			
$L_{(1-134)}B_{(136-275)}L_{(274-456)}$	2.0	1			
$L_{(1-198)}B_{(202-275)}L_{(274-456)}$	5.27 ± 5.83	3			
$L_{1-275}B_{278-456}$	38.10 ± 53.64	3			

*Two additional values for B_{CAT9} were >100.

Example 11: Mouse Serum Half-Life Assay

CD-1 mice w ighing approximately 20 grams were injected with 5 mg/kg body weight BPI, LBP, or RENPs (1 mg/ml) at time zero. In heparinized (or later
5 EDTA-containing) tubes, blood was collected from the retroorbital plexus from three animals for each time point tested. A typical blood collection schedule was 5, 10, 15, 30, 45, 60, 90, 120, 240, and 360 minutes. The EDTA anticoagulated blood was centrifuged for about
10 10 min at 1000 x g and the supernatant plasma removed and stored frozen on dry ice until tested. Levels of BPI, LBP, or RENP in the plasma samples were determined by ELISA using the appropriate protein as the standard.

A potent anti-endotoxin therapeutic should not
15 only neutralize endotoxin, but should also have the capacity to clear endotoxin from the circulation. The circulating levels of radioactively labeled ^{125}I-BPI were measured in the mouse in the presence and absence of endotoxin (Table 7). In the absence of endotoxin, the
20 elimination (alpha) phase for ^{125}I-BPI was less than two minutes. In the presence of LPS, the alpha phase was extended to 6.2 minutes. ^{125}I-LPS alone has a single phase distribution (beta) with a half-life of about 101 minutes. When ^{125}I-LPS and unlabeled BPI were
25 administered, a 6.2 minute elimination (alpha) phase was observed, indicating that elimination was remarkably facilitated by BPI.

Table 7

Serum Half-Life of BPI and LPS in the Mouse

Test Article	$t_{1/2}$alpha	$t_{1/2}$beta
^{125}I-BPI	1.6	103.0
^{125}I-BPI + LPS	6.3	72.0
^{125}I-LPS	---	101.0
^{125}I-LPS + BPI	6.2	114.0

In order to determine whether the very short circulating half-life of BPI could be extended by molecular engineering, the circulating half-lives of BPI, LBP, $B_{1-199}L_{200-456}$ and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ were compared (Figure 11). Using both labeled and unlabeled material, it was observed that the circulating half-life of BPI in the mouse is remarkably short. This may be a result of the highly cationic nature of BPI having a predicted pI of 10.6. LBP, normally present in the circulation at concentrations of 10 μg/ml, has a predicted pI of about 6.8. As expected, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (LBP-BPI chimera lacking BPI cationic residues) has a markedly longer circulating half-life than $B_{1-199}L_{200-456}$ (BPI-LBP chimera having BPI cationic residues). Figure 11 shows that $L_{1-197(I43->V)}B_{200-456(N206->D)}$ indeed has a longer half-life than BPI. $B_{1-199}L_{200-456}$, with the N-terminal domain of BPI, had an even shorter half-life than that of BPI. Thus, the N-terminal domain of BPI appears to play a major role in its short circulating half-life.

Further pharmacokinetic studies w re p rformed in which recombinant proteins of the subj ct inv ntion wer administered to CD-1 mice at a 5 mg/kg bolus dos .

Results of these experiments are shown in Figures

17A-17H. At 5 mg/kg, th circulating half lif of $B_{1-199}L_{200-456}$ was similar to that f BPI. $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and $L_{1-197}B_{200-456}$ had overlapping elimination curves and again indicating that

5 these two molecules are equivalent with respect to their biological activities. $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and B_{1-199} persisted in the circulation significantly longer than BPI or $B_{1-199}L_{200-456}$, but not as long as the serum protein LBP. Comparison of the elimination curves of

10 $L_{1-59}B_{60-456}$, $L_{1-134}B_{135-456}$ and B_{CAT9} revealed that the N-terminus of LBP plays a role in extending circulating half-life. $L_{1-59}B_{60-456}$ circulates slightly longer than BPI, and contains the least LBP sequence of any of the recombinant proteins tested (amino acid residues 1-59).

15 $L_{1-134}B_{135-456}$ was cleared somewhat more slowly, indicating that LBP amino acid residues 60-134 of LBP impart a longer circulating half-life. In contrast, the cationic residues of BPI between 134-199 shorten the half-life, since in B_{CAT9}, where the cationic residues in this region

20 were replaced with the corresponding residues of LBP, the half-life was similar to that of $L_{1-134}B_{135-456}$. Including more LBP residues in the N-terminal domain further extends the half life. If amino acid residues 199-359 of LBP are added ($L_{1-359}B_{360-456}$), the half-life is longer,

25 but not quite as long as that of LBP. Likewise $L_{(1-198)}B_{(202-275)}L_{(274-456)}$ (with LBP domain 1-198 and 274-456) has a relatively long t1/2. These results indicate that the more "LBP-like" the molecule is, the longer it circulates. In addition, combining an Ig

30 fragment Fc with $L_{1-197(I43->V)}B_{200-456(N206->D)}$ gives the longest half life.

Example 13: Mouse Endotoxin Challenge Assay

Female CD-1 mic w re inj cted in th lateral tail vein with a LD_{100} dose (25-35 mg/kg) of Salmonella abortus

equi endotoxin, which was follow d by an injecti n f
BPI, RENP, or vehicle c ntr l int the opp sit lateral
tail vein at the indicated time. Protein injection
concentrations varied and provided doses of 0.5, 1, and
5   5 mg/kg. Use of vehicle control illustrated the
lethality of the endotoxin challenge in the test animal.
Animals were observed for mortality at 24, 28, and 72
hours. Preliminary studies showed that mortality does
not change from day three to day seven or beyond.

10   The efficacies of BPI, LBP,

$L_{1-197(I43->V)}B_{200-456(N206->D)}$, $B_{1-199}L_{200-456}$ and $B_{(S351->A)}$
against lethal endotoxin challenge in mice were compared
(Tables 8-10). The efficacies of

$L_{1-197(I43->V)}B_{200-456(N206->D)}$, $L_{1-197}B_{200-456}$, $L_{1-59}B_{60-456}$,
15 $L_{1-134}B_{135-456}$, $L_{(1-198)}B_{(201-456)}FC$, $L_{1-275}B_{278-456}$,
$L_{1-359}B_{360-456}$, B_{CAT9}, B_{CAT6}, and B_{CAT15} against lethal
endotoxin challenge in mice were also compared (Table
11). When each protein was given within two minutes
after lethal endotoxin challenge, BPI,

20 $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and $B_{(S351->A)}$ had similar
potency, whereas LBP and $B_{1-199}L_{200-456}$ showed minimal
protection. The marginal protective effects of LBP and
$B_{1-199}L_{200-456}$ since these agents do not block the
inflammatory signal of LPS acting on human cells *in vitro*
25 (Figure 10).

Table 8

Mouse Endotoxin Challenge
Comparison of BPI, LBP (NCY102),
and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103)

Drug	Dose	% Survival (n=10)
Control	0 mg/kg	0%
BPI	5 mg/kg	60%
	1 mg/kg	40%
LBP	5 mg/kg	30%
	1 mg/kg	20%
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	5 mg/kg	60%
	1 mg/kg	50%

Table 9

Mouse Endotoxin Challenge
Comparison of BPI, $L_{1-197(I43->V)}B_{200-456(N206->D)}$ and
$B_{(S351->A)}$

Drug	Dose	% Survival (n=10)
Control	0 mg/kg	0%
BPI	5 mg/kg	80%
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	5 mg/kg	100%
$B_{(S351->A)}$	5 mg/kg	90%

Table 10

Mouse Endotoxin Challenge
Comparison of BPI and $B_{1-199}L_{200-456}$ (NCY104)

Drug	Dose	% Survival (n=10)
Control	0 mg/kg	40%
BPI	10 mg/kg	100%
	2 mg/kg	100%
	0.4 mg/kg	70%
$B_{1-199}L_{200-456}$	10 mg/kg	60%
	2 mg/kg	60%
	0.2 mg/kg	50%

Table 11

Survival in CD-1 Mice Following
Lethal Endotoxin Challenge

Panel A

	Survivors/n	% Survival	p (vs.control)
BPI	40/50	80.00	< 0.001
$L_{1-197(I43->V)}B_{200-456(N206->D)}$	17/20	85.00	< 0.001
$L_{1-197}B_{200-456}$	16/20	80.00	< 0.001
$L_{1-59}B_{60-456}$	13/20	65.00	< 0.001
$L_{1-134}B_{135-456}$	13/20	65.00	< 0.001
$L_{(1-198)}B_{(201-456)}Fc$	5/10	50.00	0.002
$L_{1-359}B_{360-456}$	2/10	20.00	0.149
B_{CAT6}	9/10	90.00	< 0.001
B_{CAT9}	1/10	10.00	0.442
$L_{1-275}B_{278-456}$	0/10	0	--
B_{CAT15}	6/10	60.0	< 0.05
Contr 1	1/30	3.30	--

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Panel B

	Dose mg/kg	Survivors (n=20)	% Survival	p (vs. control)*
BPI				
	5	13	65	< 0.001
	1	9	45	0.001
	0.5	6	30	0.02
$L_{1-197(I43->V)}B_{200-456(N206->D)}$				
	5	18	90	<0.001
	1	12	60	<0.001
	0.5	9	45	0.001
B_{1-199}				
	5	3	15	NS
	1	0	0	NS
	0.5	1	5	NS

* Fisher's Exact Test

$L_{1-197(I43->V)}B_{200-456(N206->D)}$ was markedly more effective than BPI when given more than an hour before or after LPS (Figure 12). These results indicate that the longer circulating half-life of

$L_{1-197(I43->V)}B_{200-456(N206->D)}$, or perhaps the increased ability of $L_{1-197(I43->V)}B_{200-456(N206->D)}$ to inhibit endotoxin in whole blood, has a dramatic effect on

$L_{1-197(I43->V)}B_{200-456(N206->D)}$ efficacy *in vivo*.

Further experiments were performed to assess the LPS-neutralizing activities of recombinant proteins of the subject invention *in vivo*. In these experiments, a lethal LPS challenge was administered at time zero, followed immediately by a 5 mg/kg bolus injection of recombinant protein.

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Example 12: BPI Reduction of LPS-Induced Cytokine
Function and Mortality in Rats

The potential effect of BPI against LPS related
cytokine formation and mortality was investigated in rats
5   with either (a) hemorrhagic shock (bled to lower pressure
to 30-35 mmHg mean arterial pressure for 90 minutes,
followed by reinfusion of shed blood and an equal volume
of Ringer's over 30 minutes), or (b) endotoxic shock
(caused by 100 μg LPS and 500 mg D-galactosamine/ kg).
10 Similarly, recombinant BPI binds LPS and inhibits TNF
formation *in vitro*. Treatment comprised 5 mg BPI/kg i.v.
for the BPI group, or 1 ml saline i.v. for the control
group.

The results of the investigation of BPI efficacy
15 in rats with either (a) hemorrhagic shock or (b)
endotoxic shock show that (a) in rats with hemorrhagic
shock, the mortality was decreased from 5/10 (50% control
group) to 2/10 (20% BPI group) at 48 hours; (b) in rats
with endotoxic shock, the 5-day mortality was
20 significantly reduced (p = 0.055) by BPI treatment to
43%, as compared to 83% in the control group. Plasma LPS
levels were at least partially neutralized at two hours
(5.9 ± 4.1 vs 10.8 ± 4.1 ng/ml). Cytokine formation was
concomitantly reduced in the BPI group as measured by
25 plasma TNF levels at two hours (3.9 ± 2.9 vs 10.3 ±
6.3 ng/ml). Liver transaminases (GOT and GPT, whose
elevation indicates hepatic dysfunction) and bilirubin
still increased at eight hours; however, the increase was
less with BPI. These data demonstrate that BPI has
30 utility as a therapeutic agent against endotoxin-related
disorders in hemorrhagic as well as endotoxic shock.

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Example 14: Protection against LPS challenge by intrapulmonary delivery of RENPs

Anesthetized male CD-1 mice were treated intra-nasally with 1 or 10 μg of either BPI or

5    $L_{1-197(I43->V)}B_{200-456(N206->D)}$ in 50 μl. Control animals received 50 μl of saline for injection. After 20 minutes, animals were re-anesthetized, and challenged with 10 ng of E. coli O55:B5 LPS. One hour after endotoxin challenge, mice were re-anesthetized, and

10   0.7 ml of saline containing 1% human serum albumin was added to the lungs via the trachea. The lungs were gently kneaded. A 0.5 ml volume of BAL (bronchoalveolar lavage) fluid was aspirated, cells were pelleted by centrifugation, and the BAL sample was sorted at -70°C.

15   The TNF-alpha level in the BAL fluid was determined by ELISA (results shown in Figure 19).

Figure 19 shows that endotoxin-neutralizing proteins such as BPI and $L_{1-197(I43->V)}B_{200-456(N206->D)}$ (NCY103) can also neutralize endotoxin-mediated TNF

20   release in the lung. These results indicate that these proteins are effective when delivered directly into the lung and thus may be useful for treatment of pneumonias and other endotoxin-related disorders of the lung, such as ARDS.

25   ## Example 15. Construction of $L_{1-197}B_{200-456}$

cDNA encoding $L_{1-197}B_{200-456}$ was constructed by creating a unique ClaI site at the junction between the nucleotide sequence coding for $Ile_{197}-Asp_{198}$ residues (ATA-GAT -> ATC-GAT). For $L_{1-197}B_{200-456}$, a 0.7 kb

30   NheI/ClaI DNA fragment (encoding amino acids 1-197) derived from the 5' sequence of LBP and a 0.8 kb ClaI/XhoI fragment (ncoding amino acids 200-456) d rived from th 3' s qu nce of BPI wer generated by PCR. The chimeric cDNAs were spliced together by cloning th

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fragments into pSE, a mammalian vector. The cDNAs for
BPI, LBP and $L_{1-197}B_{200-456}$ were transf cted into Chin s
hamster ovary cells (strain DUXB11) using lipofectin.
The resulting transformed cells were selected, and
5   expression was amplified with methotrexate. Cell culture
supernatants were screened for reactivity by ELISA.
Recombinant BPI, LBP, and $L_{1-197}B_{200-456}$ were purified as
described above.

Example 16. Pharmokinetics of $L_{1-197}B_{200-456}$
10   Data for pharmacokinetic analysis were collected
from healthy CD-1 mice given a single bolus injection
(5 mg/kg) of recombinant protein at time=0. Blood was
collected from three mice for each collection time point
by retroorbital puncture at timepoints over three hours.
15 Blood samples anticoagulated in EDTA were assayed by a
double antibody sandwich ELISA for the presence of BPI,
LBP or $L_{1-197}B_{200-456}$). Pharmacokinetic analysis was
performed using a non-compartmental analysis (PharmK
pharmacokinetic software, SoftRes, Inc.).
20   Comparison of BPI and LBP shows that BPI was
cleared rapidly with a clearance rate of 13.0 ml/minute
(Table 12). LBP had the longest half life, with a
clearance rate of 0.042 ml/min. Compared to BPI, LBP was
cleared 310 times more slowly. $L_{1-197}B_{200-456}$ had an
25 intermediate half life (Clearance rate = 0.175 ml/min),
being cleared 74 times more slowly than BPI.

Table 12: Clearance rate of $L_{1-197}B_{200-456}$

	CL (ml/min)	(vs. BPI)
BPI	13.000	-
LBP	0.042	(310 fold)
$L_{1-197}B_{200-456}$	0.175	(74 fold)

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Example 17.   LPS protection by $L_{1-197}B_{200-456}$

Female CD-1 mice (n=10) were injected in the
lateral tail vein with 35 mg/kg S. abortus equi LPS
(Sigma, St. Louis, MO) at time=0.   Recombinant protein
5   (5 mg/kg) was then administered intravenously into the
opposite lateral tail vein immediately following (t=0)
endotoxin challenge.   Survival was monitored at 24, 48
and 72 hours post-challenge.   Control animals received
0.1 ml saline instead of recombinant protein.   The p
10   values were determined by Fisher's exact test.

The results are shown in Figure 20.   BPI and
$L_{1-197}B_{200-456}$ provided 90% to 100% survival, respectively,
at the 72 hour end point.   No further mortality was noted
at seven days post-challenge.   The untreated control
15   group had a survival rate of 20%.   The survival rates of
the treated groups were statistically significant
compared to the control group (p<.001 for the
$L_{1-197}B_{200-456}$ group and p=.003 for the BPI group
determined by Fisher's exact test).   These results
20   indicate that $L_{1-197}B_{200-456}$ is as effective as BPI in this
endotoxin challenge model in vivo.

Example 18.   Protection Against Endotoxin Challenge in
Mice

The ability of the recombinant,
25   endotoxin-neutralizing proteins $B_{(1-41)}L_{(1-199)}B_{(1-456)}$,
$L_{(1-164)}B_{(200-456)}$, $B_{(1-175)}B_{(200-456)}$, $B_{(1-236)}$, and $B_{(1-190)}$ to
protect mice against endotoxin challenge was carried out
as described in Example 17 above. Protection by these
proteins was compared to the protection provided by BPI
30   or saline.   The results of these studies are shown in
Table 13.

TABLE 13

	Compound	Lot #	Number of Survivors/10 at Time (hours)							
			0	12	18	24	36	48	60	72
Group 1	native BPI	149724	10	10	10	10	10	9	9	9
Group 2	$B_{(1-41)}L_{(1-199)}B_{(1-456)}$	162303	10	10	10	10	9	9	8	8
Group 3	$L_{(1-164)}B_{(200-456)}$	164325	10	10	9	9	8	8	7	7
Group 4	$L_{(1-175)}B_{(200-456)}$	164326	10	10	10	10	10	10	10	10
Group 5	$B_{(1-236)}$	159695	8	7	5	4	1	0	0	0
Group 6	$B_{(1-190)}$	159699	10	9	8	6	6	6	5	5
Group 7	Saline		10	8	7	6	4	3	3	3

Each animal received 35 mg/kg LPS in 0.1 ml, followed immediately by 5 mg/kg of the indicated compound in 0.1 ml. Survival was monitored at each time point indicated.

Example 19:   Detection of a Gram-negative infection in a patient

A blood sample of about 1 ml to 5 ml is drawn from a patient suspected of having a Gram-negative infection. The blood sample is treated with citrate anti-coagulant and plasma is separated from the blood cells by centrifugation. The plasma is then diluted in a series of 10-fold dilutions in assay buffer (pyrogen-free TBS + 1 mg/ml low endotoxin BSA, and 0.05% Tween-20). The diluted plasma samples are then mixed with a known amount of biotinylated RENP. A series of control samples containing known amounts of biotinylated RENP in assay

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buffer is included in th assay as quantitative and
negativ controls.

The test and control samples are then applied to
the wells of a microtiter plate having bound LPS. The
5 LPS-bound microtiter wells are prepared by incubation
with 1 or 4 µg of S. minnesota R595 Re LPS (LIST
Biological Labs, Inc., #304) in 50 mM borate pH 9.5-9.8 +
20-25 mM EDTA overnight at 37°C. Blank, non-LPS coated
wells are included on each plate as controls for
10 non-specific binding. The plates are then washed
extensively under running distilled deionized water, then
dried at 37°C. The assay wells are subsequently blocked
for 60 minutes at 37°C with 1-2% very low endotoxin BSA
(Sigma, St. Louis, MO) prepared in pyrogen-free
15 Tris-buffered saline (50 mM Tris pH 7.4 + 150 mM NaCl).

The test and control samples are incubated for a
time sufficient for binding of the RENP in the samples to
the LPS bound to the microtiter wells, generally about
2-3 hours at 37°C in a total volume of 100 µl/well.
20 After incubation, the wells are washed four times with
assay buffer, and the plates are developed with
streptavidin conjugated to alkaline phosphatase followed
by 100 µl of PNP substrate solution freshly prepared from
two 5 mg tablets dissolved in 10 ml substrate buffer.
25 Substrate buffer is prepared with 24.5 mg $MgCl_2$, 48 ml
diethanolamine, brought up to 400 ml, pH adjusted to 9.8
and volume brought up to 500 ml. Absorbances are read at
405 nm on a microplate reader.

If the level of biotinylated RENP bound to the
30 wells of the test sample is significantly less than the
level of biotinylated RENP bound to the negative control
sample, then the patient has endotoxin circulating in the
bloodstream which is generally associated with a
Gram-negative inf ction. Mor ov r, th lev l of RENP
35 binding in the test sample is compar d to the l vels of

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RENP binding in the quantitativ controls, each of which
are r pres ntative f varying d grees f severity of
Gram-negative infection in a patient. The level of
binding of the test sample is thus compared to the levels
5 of binding of the quantitative samples to determine a
degree of severity of infection.

Example 20: Detection of a Gram-negative infection in
vivo

           RENP is detectably labeled with ^{125}I using methods
10 well known in the art. Approximately 100 μg of an
^{125}I-labeled RENP is injected intravenously into a patient
suspected of having a Gram-negative infection in an
organ, e.g., the liver. After allowing a time sufficient
for circulation of the ^{125}I-labeled RENP to the suspected
15 site of infection, the abdomen of the patient is
fluoroscoped or X-rayed 2 to 3 times so as to include
various perspectives. The X-ray is then examined to
identify sites of binding of the RENP by virtue of an
abnormally darkened section on the X-ray. Upon
20 identification of the site of infection, the clinician
designs an appropriate therapeutic regimen.
           Following procedures similar to those described
above, other recombinant, LPS-binding proteins can be
produced and used in diagnostic methods and methods of
25 treatment according to the invention.

           The invention now being fully described, it will
be apparent to one of ordinary skill in the art that many
changes and modifications can be made thereto without
departing from the spirit or scope of the appended
30 claims.

CLAIMS   - 87 -

1.   A m th d of detecting a sit   f Gram-n gativ
bacterial infection in a subj ct, said method comprising
the steps of:

injecting into the patient's circulatory system an
5 injectable formulation comprising an effective amount of
a recombinant endotoxin-neutralizing polypeptide attached
to a detectable label, wherein the polypeptide is
characterized by (i) selective and specific binding to
lipopolysaccharide and (ii) endotoxin-neutralizing
10 activity, with the proviso that the amino acid sequence
of the polypeptide is not identical to the amino acid
sequences of BPI or LBP;

allowing the detectably labeled polypeptide
sufficient time to circulate in the subject and bind to
15 lipopolysaccharide in the patient; and

detecting a site of label binding in the patient,
thereby detecting a site of Gram-negative bacterial
infection.

2.   The method of claim 1, wherein the
20 polypeptide is covalently bound to a molecule which
enhances the half-life of the polypeptide.

3.   The method of claim 1, wherein the
polypeptide contains an LPS binding domain of BPI, LBP, a
BPI variant, or an LBP variant.

25   4.   The method of claim 1, wherein the detectable
label is a radionucleotide.

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5.   A method of detecting a Gram-n gative
bacterial infecti n in a subj ct, said meth d comprising
the steps of:
      obtaining a sample from a patient suspected of
5 having a Gram-negative bacterial infection;
      contacting said sample with a detectably labeled
recombinant endotoxin-neutralizing polypeptide for a time
sufficient for binding of the polypeptide to
lipopolysaccharide in the sample; and
10   detecting formation of
lipopolysaccharide-polypeptide complexes by detection of
a detectable label bound to the polypeptide;
      wherein detection of a level of detectable label
in said sample significantly greater than a level of
15 detectable label in a negative control sample is
indicative of a Gram-negative bacterial infection in the
subject.

6.   The method of claim 5, wherein the
polypeptide contains an LPS binding domain of BPI, LBP, a
20 BPI variant, or an LBP variant.

7.   The method of claim 5, wherein said detection
is quantitative.

8.   The method of claim 7, wherein said
quantitative detection is correlated with an
25 Gram-negative bacterial infection load.

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9.   A detectably labeled recombinant
end toxin-neutralizing p lyp ptid characterized by (i)
selective and specific binding to lipopolysaccharide and
(ii) endotoxin-neutralizing activity, with the proviso
5 that the amino acid sequence of the polypeptide is not
identical to the amino acid sequences of BPI or LBP.

10.   A polypeptide according to claim 9, wherein
the polypeptide contains an LPS binding domain of BPI,
LBP, a BPI variant, or an LBP variant.

10   11.   A detectably labeled polypeptide according to
claim 9, wherein the polypeptide comprises a molecule
which enhances the half-life of said polypeptide and is
covalently bound to the polypeptide.

12.   A detectably labeled polypeptide according to
15 claim 11, wherein said molecule is an immunoglobulin
fragment, a half-life enhancing porion of LBP, a
half-life enhancing portion of an LBP variant, or
polyethylene glycol.

13.   A kit for detecting a site of Gram-negative
20 bacterial infection in a subject, the kit comprising:
        an injectable formulation comprising a detectably
labeled recombinant endotoxin-neutralizing polypeptide
characterized by (i) selective and specific binding to
lipopolysaccharide and (ii) endotoxin-neutralizing
25 activity, with the proviso that the amino acid sequence
of the polypeptide is not identical to the amino acid
sequences of BPI or LBP.

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14.   A recombinant ndotoxin-neutralizing
polyp ptide characterized by (i) selective and specific
binding to lipopolysaccharide and
(ii) endotoxin-neutralizing activity, with the proviso
5 that the amino acid sequence of the polypeptide is not
identical to the amino acid sequences of BPI or LBP.

15.   A recombinant endotoxin-neutralizing
polypeptide according to claim 14, wherein said
polypeptide is of the formula $L_{1-197}B_{200-456}$ or a
10 corresponding protein which (a) functions to bind
lipopolysaccharide and (b) neutralizes endotoxin.

16.   A recombinant endotoxin-neutralizing
polypeptide according to claim 14, wherein the
polypeptide is BPI(S351->X), wherein X is any amino acid
15 other than serine.

17.   A recombinant endotoxin-neutralizing
polypeptide according to claim 16, wherein X is alanine.

18.   A recombinant endotoxin-neutralizing
polypeptide according to claim 14, wherein the
20 polypeptide contains the amino acid sequence of BPI
having a cationic amino acid substituted with a neutral
or anionic residue.

19.   A recombinant endotoxin-neutralizing
polypeptide according to claim 18, wherein the cationic
25 amino acid is at BPI amino acid residue positions 27, 30,
33, 42, 44, 48, 59, 77, 86, 90, 96, 118, 127, 148, 150,
160, 161, 167, 169, 177, 185, or 198.

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20. A recombinant ndotoxin-neutralizing polyp ptide according t claim 19, wh rein the polypeptide contains neutral or anionic residues at BPI amino acid residue positions 27, 30, 33, 42, 44, 48, and
5 59.

21. A recombinant endotoxin-neutralizing polypeptide according to claim 19, wherein the polypeptide contains neutral or anionic residues at BPI amino acid residue positions 77, 86, 90, 96, 118, and
10 127.

22. A recombinant endotoxin-neutralizing polypeptide according to claim 19, wherein the polypeptide contains neutral or anionic residues at BPI amino acid residue positions 148, 150, 160, 161, 167,
15 169, 177, 185, and 198.

23. A recombinant endotoxin-neutralizing polypeptide according to claim 18, wherein the polypeptide contains neutral or anionic residues at BPI amino acid residue positions 27, 30, 33, 42, 44, 48, 59,
20 77, 86, 90, 96, 118, 127, 148, 150, 160, 161, 167, 169, 177, 185, and 198.

24. A recombinant endotoxin-neutralizing polypeptide according to claim 14, wherein the polypeptide contains the amino acid sequence of LBP
25 having an amino acid substituted for an amino acid in a corresponding amino acid residue position of BPI.

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25.   A recombinant endotoxin-neutralizing
p lypeptide according to claim 24, wherein th amino acid
substituted is at LBP amino acid residue positions 77,
86, 96, 118, 126, 147, 148, 158, 159, 161, 165, 167, 175,
5 183, or 196.

26.   A recombinant endotoxin-neutralizing
polypeptide according to claim 14, wherein the
polypeptide contains the amino acid sequence of BPI
having a cysteine residue substituted with an amino acid
10 other than cysteine.

27.   A recombinant endotoxin-neutralizing
polypeptide according to claim 26, wherein said cysteine
residue is at BPI amino acid residue position 132, 135,
or 175.

15   28.   A recombinant endotoxin-neutralizing
polypeptide according to claim 26, wherein the cysteine
residues of BPI at positions 132, 135, and 175 are
substituted with an amino acid other than cysteine.

29.   A recombinant endotoxin-neutralizing
20 polypeptide according to claim 14, wherein the
polypeptide comprises a molecule which enhances the
half-life of said polypeptide and is covalently bound to
the polypeptide.

30.   A recombinant endotoxin-neutralizing
25 polypeptide according to claim 29, wherein said
polypeptide contains a lipopolysaccharide-binding domain
of BPI, LBP, a BPI variant, or an LBP variant.

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31. A recombinant ndotoxin-neutralizing
polypeptide acc rding t claim 29, wherein said molecule
is an immunoglobulin fragment, a half-life enhancing
portion of LBP, a half-life enhancing portion of an LBP
5 variant, or polyethylene glycol.

32. A recombinant endotoxin-neutralizing
polypeptide according to claim 29, wherein the
endotoxin-neutralizing polypeptide of (a) is a C-terminal
fragment of BPI and the molecule of (b) is an N-terminal
10 fragment of LBP.

33. A recombinant endotoxin-neutralizing
polypeptide according to claim 32, wherein said
C-terminal fragment of BPI is a fragment having an amino
acid sequence contained in BPI amino acid residues
.15 60-456.

34. A recombinant endotoxin-neutralizing
polypeptide according to claim 33, wherein said
C-terminal fragment of BPI is BPI amino acid residues
60-456, 136-456, 277-456, 300-456, 200-456, 136-361,
20 136-275, 200-275, or 200-361.

35. A recombinant endotoxin-neutralizing
polypeptide according to claim 32, wherein said
N-terminal fragment of LBP is a fragment having an amino
acid sequence contained in LBP amino acid residues 1-175.

25   36. A recombinant endotoxin-neutralizing
polypeptide according to claim 21, wherein said
N-terminal fragment of LBP is LBP amino acid residues
1-59, 1-134, 1-164, 1-175, 1-274, 1-359, 1-134, or 1-197.

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37.   A recombinant ndot xin-n utralizing
polypeptide of claim 18, wherein the p lypeptide further
comprises a C-terminal fragment of LBP.

38.   A recombinant endotoxin-neutralizing
5 polypeptide of claim 23, wherein the C-terminal fragment
of LBP is LBP amino acid residues 360-456 or 274-456.

39.   An isolated DNA molecule encoding a
recombinant endotoxin binding polypeptide according to
claim 14.

10   40.   A vector comprising the DNA of claim 39.

41.   A transformed host cell comprising the DNA of
claim 39.

42.   A method for producing a recombinant
endotoxin-neutralizing polypeptide according to claim 14,
15 said method comprising the steps of:
        culturing a transformed host cell comprising DNA
encoding a recombinant endotoxin binding polypeptide
according to claim 14, said DNA being operably linked to
a promoter for expression of the polypeptide encoded by
20 the DNA, said culturing being under conditions allowing
expression of said polypeptide; and
        isolating the recombinant endotoxin binding
polypeptide produced.

43.   A pharmaceutical composition comprising:
25   a therapeutically effective amount of a
recombinant endotoxin-neutralizing polypeptide according
to claim 14; and
        a pharmaceutically acc ptable carri r.

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44. A m thod f tr ating a subject suffering from
an endotoxin-r lat d disorder, said meth d comprising:
administering to a subject having an
endotoxin-related disorder a therapeutically effective
5 amount of a recombinant endotoxin-neutralizing
polypeptide according to claim 14, wherein LPS-mediated
stimulation of neutrophils and mononuclear cells is
inhibited.

45. A method of preventing an endotoxin-related
10 disorder in a subject, said method comprising:
administering to a subject a prophylactically
effective amount of a recombinant endotoxin-neutralizing
polypeptide according to claim 14, wherein the
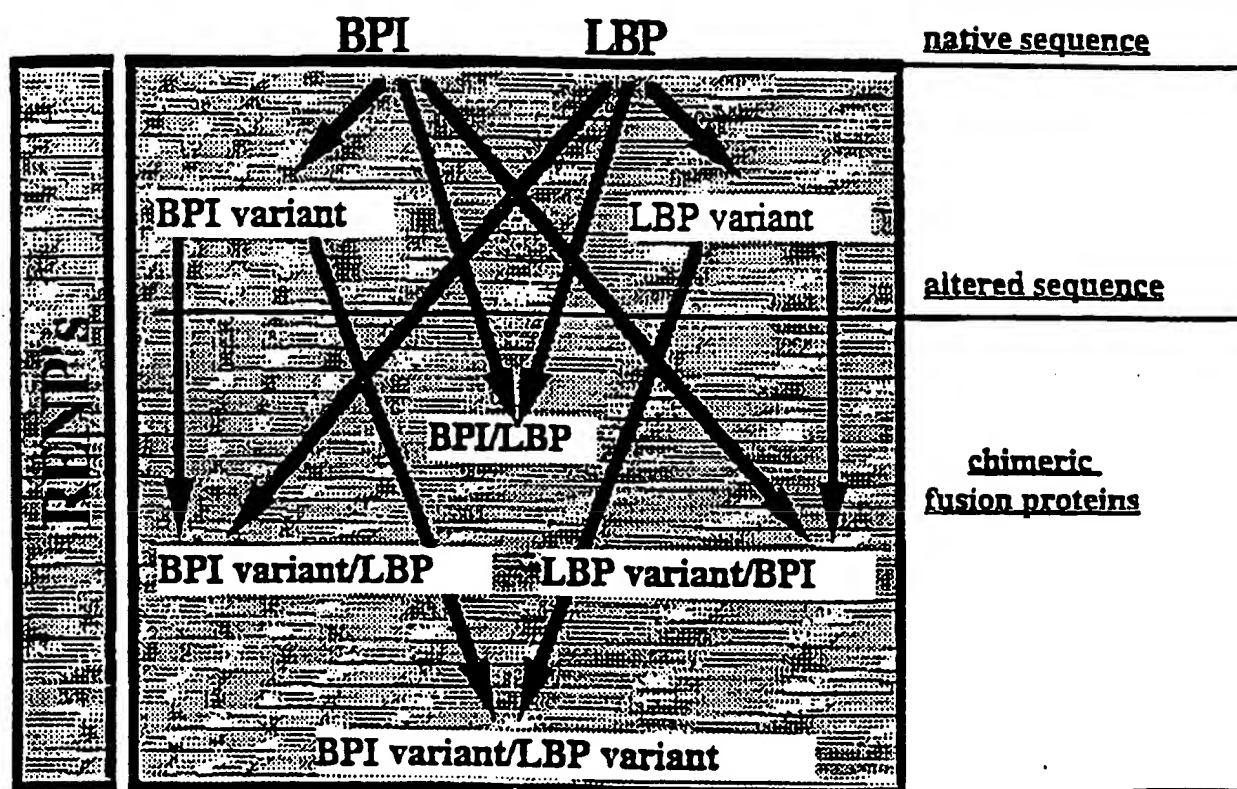endotoxin-related disorder is prevented.

FIGURE 1A

| FIGURE 1A |
| FIGURE 1B |
| FIGURE 1C |
| FIGURE 1D |

Human LBP-a (as reported by Schumann et al.)
Human LBP-b (correct sequence)

```
                              -25
                        m
                        m

       -20          -10              -1
Human LBP-a  g a l a h a l p s i - - - - i s t p e a l r
Human LBP-b  g a l a h a l p s i - - - - i s t p e a l r

                     10                  20
Human LBP-a  A N P G L V A R I T D K G L Q Y A A Q E G L L A L
Human LBP-b  A N P G L V A R I T D K G L Q Y A A Q E G L L A L

             30              40                  50
Human LBP-a  Q S E L R I T L P D F T G D L R I P H V G R G R
Human LBP-b  Q S E L R I T L P D F T G D L R I P H V G R G R

             60              70
Human LBP-a  Y F H S L N I H S C E L L H S A L R P V P G Q G
Human LBP-b  Y F H S L N I H S C E L L H S A L R P V P G Q G
```

FIGURE 1B

```
                     80              90           100
Human  IBP-a   L S L S I S D S S I R V Q G R W K V R K S F F K I
Human  IBP-b   L S L S I S D S S I R V Q G R W K V R K S F F K I

                            110         120
               Q G S F D V S V K G I S V N L L G S E S
               Q G S F D V S V K G I S V N L L G S E S

                       130         140
Human  IBP-a   G R P T G C Y L S C C S D I A D V E V D M S G D
Human  IBP-b   G R P T V T A S S C S S D I A D V E V D M S G D

                   150         160         170
               S G W L L N L F H N Q I E S K F Q K V L E S R I C
               F G W L L N L F H N Q I E S K F Q K V L E S R I C

                           180         190      25k/30k
Human  IBP-a   E M I Q K S V S S D L Q P Y L Q T L P V T T E I D
Human  IBP-b   E M I Q K S V S S D L Q P Y L Q T L P V T T E I D
```

FIGURE 1C

```
                200         210         220
IBP-a    * S F A D I D Y S L V E A P R A T A Q M L E V M F K
IBP-b      S . A D I D Y S L V E A P R A T A Q M L E V M F K

                230         240
IBP-a    G E I F H R N H R S P V T L L A A A . . . E E H
IBP-b    G E . F H R N H R S P V T L L A A V . . . E E H

                250         260         270
IBP-a    N K M V Y F A I S D Y V F N T A S L V Y H E E G Y
IBP-b    N K N V Y Y A I S D Y V F N T A S L V Y H E E G Y

                280         290
IBP-a    L N F S I T D D M I P P D S N I R L T K S F R R P
IBP-b    L N F S I T D D M I P P D S N I R L T K S F R R P

                300         310         320
IBP-a    F V P R L A R L Y P N M N L E L Q G S V P S A P L
IBP-b    F V P R L A R L Y P N M N L E L Q G S V P S A P L
```

FIGURE 1D

```
                    330           340              350
LBP-a   L N F S P G N L S V D P Y M E I D A F V L L P S
LBP-b   L N F S P G N L S V D P Y M E I D A F V L L P S

                    360           370
LBP-a   S K E P V F R L S V A T N V S A T L F N T S K I
LBP-b   S K E P V F R L S V A T N V S A T L T N T S K I

                    380           390              400
LBP-a   T G F L K P G K V K V E L K E S K V G L E N A L I
LBP-b   T G F L K P G K V K V E L K E S K V G L E N A L L

                    410           420
LBP-a   L E A L L N Y Y I L N T L Y P K F N D K L A E G F
LBP-b   L E A L L N Y Y I L N T F Y P K F N D K L A E G F

                    430           440
LBP-a   P L P L L K R V Q L Y D L G L Q I H K D L
LBP-b   P L P L L K R V Q L Y D L G L Q I H K D L

        450           456
LBP-a   A N V Q Y M R V
LBP-b   A N V Q Y M R V
```

Figure 2

FIGURE 3A

FIGURE 3A
FIGURE 3B
FIGURE 3C
FIGURE 3D

```
I cDNA

1    CAG GCC TTG AGG TTT TGG CAG CTC TGG AGG ATG AGA GAG AAC ATG GCC    48
1                                            Met Arg Glu Asn Met Ala     6

49   AGG GGC CCT TGC AAC GCG CCG AGA TGG GTG TCC CTG GTG CTC GTC        96
7    Arg Gly Pro Cys Asn Ala Pro Arg Trp Val Ser Leu Val Leu Val        22

97   GCC ATA GGC ACC GCC GTG ACA GCC GTC AAC GCC GTC GGG CAG ACG GTG   144
23   Ala Ile Gly Thr Ala Val Thr Ala Val Asn Ala Val Gly Gln Thr Val    38

145  AGG ATC TCC CAG AAG CTG GGC TAC GCC AGC CAG GGG ACG GCC           192
39   Arg Ile Ser Gln Lys Leu Gly Tyr Ala Ser Gln Gly Thr Ala            54

193  GCT CTG AAG GAG CTG AAG ATC ATT CCT GAC TCA GAC                   240
55   Ala Leu Lys Glu Leu Lys Ile Ile Pro Asp Ser Asp                    70

241  AGC TTT AAG ATC AAG CAT CTT GGG AAG TTC TAC AGC                   288
71   Ser Phe Lys Ile Lys His Leu Gly Lys Phe Tyr Ser                    86

289  ATG GAC ATC CGT GAA TTC CAG CTT CCC AGT TCC CAG ATA AGC ATG GTG   336
87   Met Asp Ile Arg Glu Phe Gln Leu Pro Ser Ser Gln Ile Ser Met Val   102
```

FIGURE 3B

```
337  CCC AAT GTG GGC CTT AAG TTC TCC ATC AGC AAC GCC AAT ATC AAG ATC  384
103  Pro Asn Val Gly Leu Lys Phe Ser Ile Ser Asn Ala Asn Ile Lys Ile  118

385  AGC GGG AAA TGG AAG AGA TTC CAA AAG GCA TTA AAA ATG AGC GGC AAT  432
119  Ser Gly Lys Trp Lys Arg Phe Gln Lys Ala Leu Lys Met Ser Gly Asn  134

433  TTT GAC CTG AGC ATA GAA GGC ATG TCC ATT TCG GCT GAT CTG AAG CTG  480
135  Phe Asp Leu Ser Ile Glu Gly Met Ser Ile Ser Ala Asp Leu Lys Leu  150

481  GGC AGT AAC CCC ACG TCA GGC AAG CCC ACC ATC ACC TGC TCC AGC TGC  528
151  Gly Ser Asn Pro Thr Ser Gly Lys Pro Thr Ile Thr Cys Ser Ser Cys  166

529  AGC AGC CAC ATC AAC AGT GTC CAC GTG CAC ATC TCA AAG AGC AAA GTC  576
167  Ser Ser His Ile Asn Ser Val His Val His Ile Ser Lys Ser Lys Val  182

577  GGG TGG CTG ATC CAA CTC TTC CAC AAA AAA ATT GAG TCT GCG CTT CGA  624
183  Gly Trp Leu Ile Gln Leu Phe His Lys Lys Ile Glu Ser Ala Leu Arg  198

625  AAC AAG ATG AAC AGC CAG GTC TGC GAG AAA GTG ACC AAT TCT GTA TCC  672
199  Asn Lys Met Asn Ser Gln Val Cys Glu Lys Val Thr Asn Ser Val Ser  214

673  TCC AAG CTG CAA CCT TAT TTC CAG CTG CCA GTA ATG ACC AAA ATA     720
215  Ser Lys Leu Gln Pro Tyr Phe Gln Leu Pro Val Met Thr Lys Ile     230

721  GAT TCT GTG GCT GGA ATC AAC TAT GGT CTG GTG GCA CCT CCA GCA ACC  768
231  Asp Ser Val Ala Gly Ile Asn Tyr Gly Leu Val Ala Pro Pro Ala Thr  246
```

FIGURE 3C

```
769  ACG GCT GAG ACC CTG GAT GTA CAG ATG AAG GGG GAG TTT TAC AGT GAG  816
247  Thr Ala Glu Thr Leu Asp Val Gln Met Lys Gly Glu Phe Tyr Ser Glu  262

817  AAC CAC CAC AAT CCA CCT TTT GCT CCA CCA GTG ATG GAG TTT CCC      864
263  Asn His His Asn Pro Pro Phe Ala Pro Pro Val Met Glu Phe Pro      278

865  GCT GCC CAT GAC CGC ATG TAC CTG GGC CTC TCA GAC TAC TTC TTC      912
279  Ala Ala His Asp Arg Met Tyr Leu Gly Leu Ser Asp Tyr Phe Phe      294

913  AAC ACA GCC GGG CTT GTA TAC CAA GAG GCT GGG GTC TTG AAG ATG ACC  960
295  Asn Thr Ala Gly Leu Val Tyr Gln Glu Ala Gly Val Leu Lys Met Thr  310

961  CTT AGA GAT GAC ATG ATT CCA AAG GAG TCC AAA TTT CGA CTG ACA     1008
311  Leu Arg Asp Asp Met Ile Pro Lys Glu Ser Lys Phe Arg Leu Thr      326

1009 AAG TTT GGA ACC CTA TTC CCT GAG GTG GCC AAG AAG TTT CCC AAC     1056
327  Lys Phe Gly Thr Leu Phe Pro Glu Val Ala Lys Lys Phe Pro Asn      342

1057 ATG AAG ATA CAG ATC CAT GTC TCA GCC ACC TAC CCC CAC CTG TCT     1104
343  Met Lys Ile Gln Ile His Val Ser Ala Thr Tyr Pro His Leu Ser      358

1105 GTG CAG CAG CCC ACC TAC CCT GCC GTC GAT GTC CAG GCC             1152
359  Val Gln Gln Pro Thr Tyr Pro Ala Val Asp Val Gln Ala             374

1153 CTT GCC GTC CTC TCC GCT TCC CTC TTC CTG ATT GGC                 1200
375  Leu Ala Val Leu Ser Ala Ser Leu Phe Leu Ile Gly                 390

1201 ATG CAC ACA ACT GGT TCC ATG GAG GTC AGC GCC GAG TCC AAC AGG CTT 1248
391  Met His Thr Thr Gly Ser Met Glu Val Ser Ala Glu Ser Asn Arg Leu 406
```

FIGURE 3D

```
249  GTT GGA GAG CTC AAG CTG GAT AGG CTC CTG GAA CTG AAG CAC TCA        1296
407  Val Gly Glu Leu Lys Leu Asp Arg Leu Leu Glu Leu Lys His Ser         422

297  AAT ATT GGC CCC TTC GTT CCG GTT TTG CTG CAG GAT ATC ATG AAC TAC     1344
421  Asn Ile Gly Pro Phe Val Pro Val Leu Leu Gln Asp Ile Met Asn Tyr     438

345  ATT GTA CCC ATT CTT GTG CTG AGA GTC AAC GAG AAA CTA CAG AAA         1392
439  Ile Val Pro Ile Leu Val Leu Arg Val Asn Glu Lys Leu Gln Lys         454

391  GGC TTC CCT CTC CCG ACG CCG GTT AGG GTC TAC AAC GTA GTG             1440
455  Gly Phe Pro Leu Pro Thr Pro Val Arg Val Tyr Asn Val Val             470

441  CTT CAG CCT CTC CAG CCT TTC AAC TTC CTG CTG GGT GCA GAC GTT GTC TAT 1488
471  Leu Gln Pro Leu Gln Pro Phe Asn Phe Leu Leu Gly Ala Asp Val Val Tyr 486

189  AAA TGA AGG CAC CAG GGG CTG TCA GCC GCA CCT GTT CCT                 1516
187  Lys ***                                                             488

537  GAT GGG CTG TGG GGC ACC GGC CTT TCC CCA GGG AAT CCT CTC CAG         1584

585  ATC TTA ACC AAG AGC CCC TTG CAA ACT TCG ACT ATT CAG AAA             1632

633  TGA TCT AAA CAC GAG GAA ACA TTA TTC ATT GGA AAA GTG CAT GGT GTG     1680

681  TAT TTT AGG GAT TAT GAG CTT CTT TCA AGG GCT AAG GCT GCA GAG ATA     1728

729  TTT CCT CCA GGA ATC GTG TTT CAA TTG TAA CCA AGA AAT TTC CAT TTG     1776

777  TGC TTC ATG AAA AAA AAC TTC TGG TTT TTT TCA TGT G                   1813
```

FIGURE 4A

FIGURE 4A
FIGURE 4B
FIGURE 4C

Human LBP   Expression clone

```
  1   GCT AGC CCA CTG CAC TGG GAA TCT AGG ATG GGG GCC TTG GCC AGA GCC    48
  1       NheI                                    Met Gly Ala Leu Ala Arg Ala     7

 49   CTG CCG TCC ATA CTG CTG GCA TTG CTG CTT ACC CCA GAG GCT            96
  8   Leu Pro Ser Ile Leu Leu Ala Leu Leu Leu Thr Pro Glu Ala           23

 97   CTG GGT GCC AAC CCC GGC TTG GTC GCC AGG ATC ACC GAC AAG GGA CTG   144
 24   Leu Gly Ala Asn Pro Gly Leu Val Ala Arg Ile Thr Asp Lys Gly Leu   39

145   CAG TAT GCG GCC CAG GAG GGG CTA TTG GCT CTG CAG AGT GAG CTC       192
 40   Gln Tyr Ala Ala Gln Glu Gly Leu Leu Ala Leu Gln Ser Glu Leu       55

193   AGG ATC ACG CTG CCT GAC TTC ACC GGG GAC TTG AGG ATC CCC CAC GTC   240
 56   Arg Ile Thr Leu Pro Asp Phe Thr Gly Asp Leu Arg Ile Pro His Val   71

241   GGC CGT TAT CGC TAT GAG TTC CAC AGC CTG AAC ATC AGC TGT GAG       288
 72   Gly Arg Tyr Arg Tyr Glu Phe His Ser Leu Asn Ile Ser Cys Glu       87

289   CTT CAC TCT GCG CTG AGG CCT GTC CCT GGC CAG GGC CTG AGT CTC       336
 88   Leu His Ser Ala Leu Arg Pro Val Pro Gly Gln Gly Leu Ser Leu      103

337   AGC ATC TCC GAC TCC ATC CGG GTC CAG GGC AGG TGG AAG GTG CGC       384
104   Ser Ile Ser Asp Ser Ile Arg Val Gln Gly Arg Trp Lys Val Arg      119

385   AAG TTC TTC AAA CTA CAG GGC TCC TTT GAT GTC AGT GTC AAG GGC       432
120   Lys Phe Phe Lys Leu Gln Gly Ser Phe Asp Val Ser Val Lys Gly      135

433   ATC AGC ATT TCG GTC AAC CTC CTG GGC TTG TTG AGC GAG TCC GGG AGG   480
136   Ile Ser Ile Ser Val Asn Leu Leu Gly Leu Leu Ser Glu Ser Gly Arg  151
```

FIGURE 4B

```
481  CCC ACA GTT ACT GCC TCC AGC TGC AGC AGT GAC ATC GCT GAC GTG GAG   528
152  Pro Thr Val Thr Ala Ser Ser Cys Ser Ser Asp Ile Ala Asp Val Glu  167

529  GTG GAC ATG TCG GGA GAC TTC GGG TGG CTG TTG AAC CTC TTC CAC AAC   576
168  Val Asp Met Ser Gly Asp Phe Gly Trp Leu Leu Asn Leu Phe His Asn  183

577  CAG ATT GAG TCC AAG TTC CAG AAA GTA CTG GAG AGC AGG ATT TGC GAA   624
184  Gln Ile Glu Ser Lys Phe Gln Lys Val Leu Glu Ser Arg Ile Cys Glu  199

625  ATG ATC CAG AAA TCG GTG TCC GAT CTA CAG CCT TAT CTC CTA CAA ACT   672
200  Met Ile Gln Lys Ser Val Ser Asp Leu Gln Pro Tyr Leu Leu Gln Thr  215

673  CTG CCA ACA ACA GAG ATT GAC AGT GCC TTC GAT ATT GAT TAT AGC      720
216  Leu Pro Thr Thr Glu Ile Asp Ser Ala Phe Asp Ile Asp Tyr Ser     231

721  TTA GTG GAA GCC CCT CGG GCA ACA GCC CAG ATG CTG GAG GTG ATG TTT   768
232  Leu Val Glu Ala Pro Arg Ala Thr Ala Gln Met Leu Glu Val Met Phe  247

769  AAG GGT GAA ATC TTT CAT CGT AAC CAC CGT TCT CCA GTT ACC CTC CTT   816
248  Lys Gly Glu Ile Phe His Arg Asn His Arg Ser Pro Val Thr Leu Leu  263

817  GCT GCA GTC ATG AGC CTT CCT GAG GAA CAC AAC AAA ATG GTC TAC TTT   864
264  Ala Ala Val Met Ser Leu Pro Glu Glu His Asn Lys Met Val Tyr Phe  279

865  GCC ATC TCG GAT GTC TAT TTC GTT AAC ACG GCC AGC CTG GTT TAT CAT GAG  912
280  Ala Ile Ser Asp Val Tyr Phe Val Asn Thr Ala Ser Leu Val Tyr His Glu 295

913  GAA GGA TAT CTG AAC TTC TCC ATC ACA GAT GAC ATG ATA CCG CCT GAC   960
296  Glu Gly Tyr Leu Asn Phe Ser Ile Thr Asp Asp Met Ile Pro Pro Asp  311

961  TCT AAT ATC CGA CTG ACC ACC AAG TCC TTC CGA GTC CCC TTC CCA CGG   1008
312  Ser Asn Ile Arg Leu Thr Thr Lys Ser Phe Arg Val Pro Phe Pro Arg  327
```

FIGURE 4C

```
1009  TTA GCC AGG CTC TAC CCC AAC ATG AAC CTG GAA CTC CAG GGA TCA GTG  1056
 328  Leu Ala Arg Leu Tyr Pro Asn Met Asn Leu Glu Leu Gln Gly Ser Val   343

1057  CCC TCT GCT CCG AAC TTC AGC CCT GGG AAT CTG TCT GTG GAC           1104
 344  Pro Ser Ala Pro Asn Phe Ser Pro Gly Asn Leu Ser Val Asp           359

1105  CCC TAT ATG GAG ATA GAT GCC TTT GTG CTC CTG CCC AGC TCC AAG       1152
 360  Pro Tyr Met Glu Ile Asp Ala Phe Val Leu Leu Pro Ser Ser Lys       375

1153  GAG CCT CGG TTC GTC AGT GTG GCC ACT AAT GTG TCC GCC ACC TTG       1200
 376  Glu Pro Arg Phe Val Ser Val Ala Thr Asn Val Ser Ala Thr Leu       391

1201  ACC AAT TTC AGC ACC AAG ATC ACT GGG TTC CTG AAG CCA GGA GTA       1248
 392  Thr Asn Phe Ser Thr Lys Ile Thr Gly Phe Leu Lys Pro Gly Val       407

1249  AAA GAA GTG CTG TCC AAA GTT GGA TCC CTA TTC AAT GCA GAG CTG       1296
 408  Lys Glu Val Leu Ser Lys Val Gly Ser Leu Phe Asn Ala Glu Leu       423

1297  TTG GAA GCG GCA TAT AAC CTC CTC AAC TAC ATC ACC AAC ACC TTC CCC AAG  1344
 424  Leu Glu Ala Ala Tyr Asn Leu Leu Asn Tyr Ile Thr Asn Thr Phe Pro Lys  439

1345  TTC AAT GAT AAG GCC GAA GGC TTG GCC GAG TTG CCC CCT CTT CTG AAG CGT  1392
 440  Phe Asn Asp Lys Ala Glu Gly Leu Ala Glu Leu Pro Pro Leu Leu Lys Arg  455

1393  GTT CAG CTC TAC GAC CTT GGG CTG CAG ATC CAT AAG GAC TTC CTG TTC  1440
 456  Val Gln Leu Tyr Asp Leu Gly Leu Gln Ile His Lys Asp Phe Leu Phe  471

1441  TTG GGT GCC AAT GTC TAC ATG TAC CAA AAT GTC GTT GGA CAA GAA AGA TGA  1488
 472  Leu Gly Ala Asn Val Tyr Met Tyr Gln Asn Val Val Gly Gln Glu Arg ***  482

1489  AGC TTG CTC GAG                                                     1500
              XhoI
```

FIGURE 5A

FIGURE 5A
FIGURE 5B
FIGURE 5C
FIGURE 5D
FIGURE 5E
FIGURE 5F

FIGURE 5B

	mouse	LBP
	rabbit	LBP
	human	LBP
	human	BPI
	ovine	BPI

FIGURE 5C

FIGURE 5D

210   220   230   240   250   260   270   280   290   300

LBP
LBP
BPI
BPI

rabbit
human
human
ovine

FIGURE 5E

FIGURE 5F

FIGURE 6

```
            10         20         30         40         50         60
   MGALARALPS ILLALLLTBT PEALGANPGL VARITDKGLQ YAAQEGLLAL QSELLRITLP
            70         80         90        100        110        120
   DFTGDLRIPH VGRGRYEFHS LNIHSCELLH SALRPVPGQG LSLSISDSSI RVQGRWKVRK
           130        140        150        160        170        180
   SFFKLQGSFD VSVKGISISV NLLIGSESSG RPTVTASSCS SDIADVEVDM SGDLGWLLNL
           190        200        210        220      25/30 230        240
   FHNQIESKFQ KVLESRICEM IQKSVSSDLQ PYLQTLPVTT EIDSVAGINY GLVAPPATTA
           250        260        270        280        290        300
   ETLDVQMKGE FYSENHHNPP PFAPPVMEFP AAHDRMVYLG LSDYFFNTAG LVYQEAGVLK
           310        320        330        340        350        360
   MTLRDDMIPK ESKFRLTTKF FGTFLPEVAK KFPNMKIQIH VSASTPPHLS VQPTGLTFYP
           370        380        390        400        410        420
   AVDVQALAVL PNSSLASLFL IGMHTTGSME VSAESNRLVG ELKLDRLLLE LKHSNIGPFP
           430        440        450        460        470        479
   VELLQDIMNY IVPILVLPRV NEKLQKGFPL PTPARVQLYN VVLQPHQNFL LFGADVVYK* -C
```

FIGURE 7

- ● BPI
- ○ LBP (NCY102)
- ■ $L_{1-197(I43\rightarrow V)}B_{200-456(N206\rightarrow D)}$ (NCY103)
- ▲ $B_{1-199}L_{200-456}$ (NCY104)

FIGURE 8

- BPI
- LBP (NCY102)
- $L_{1-197(I43 \to V)} B_{200-456(N206 \to D)}$ (NCY103)
- $B_{1-199} L_{200-456}$ (NCY104)
- $B_{(S351 \to A)}$ (NCY105)
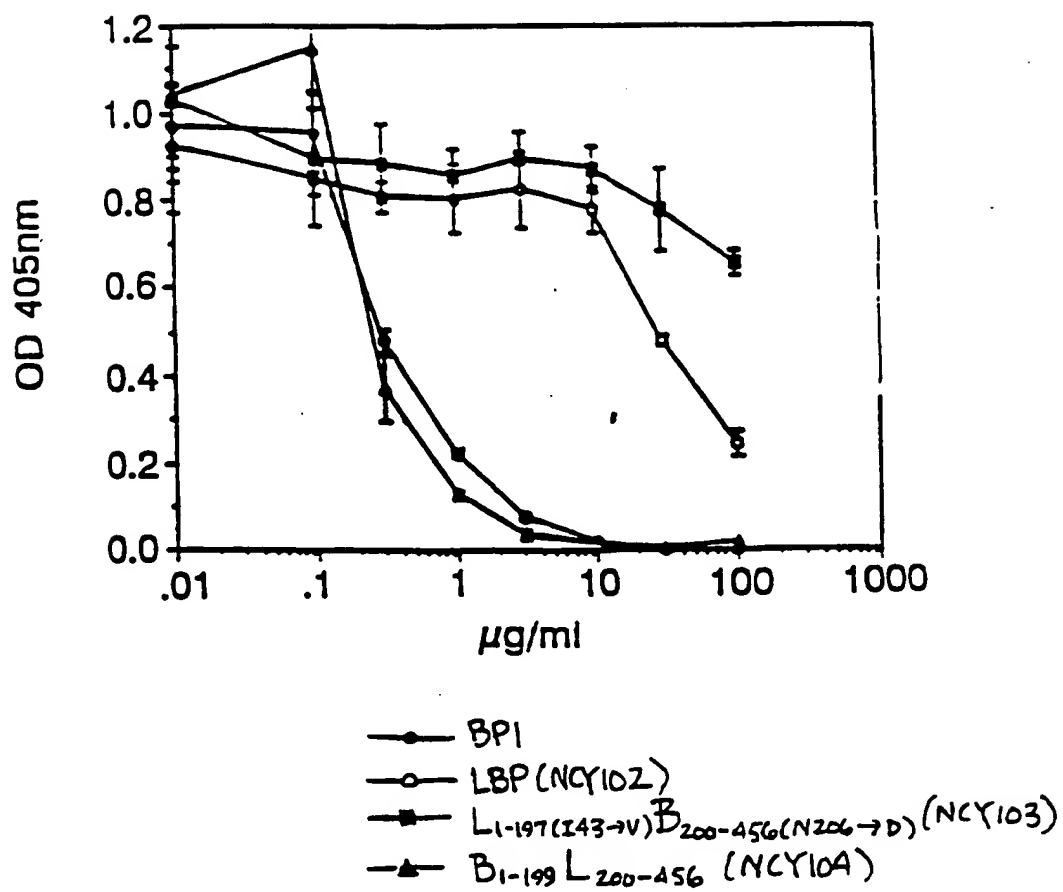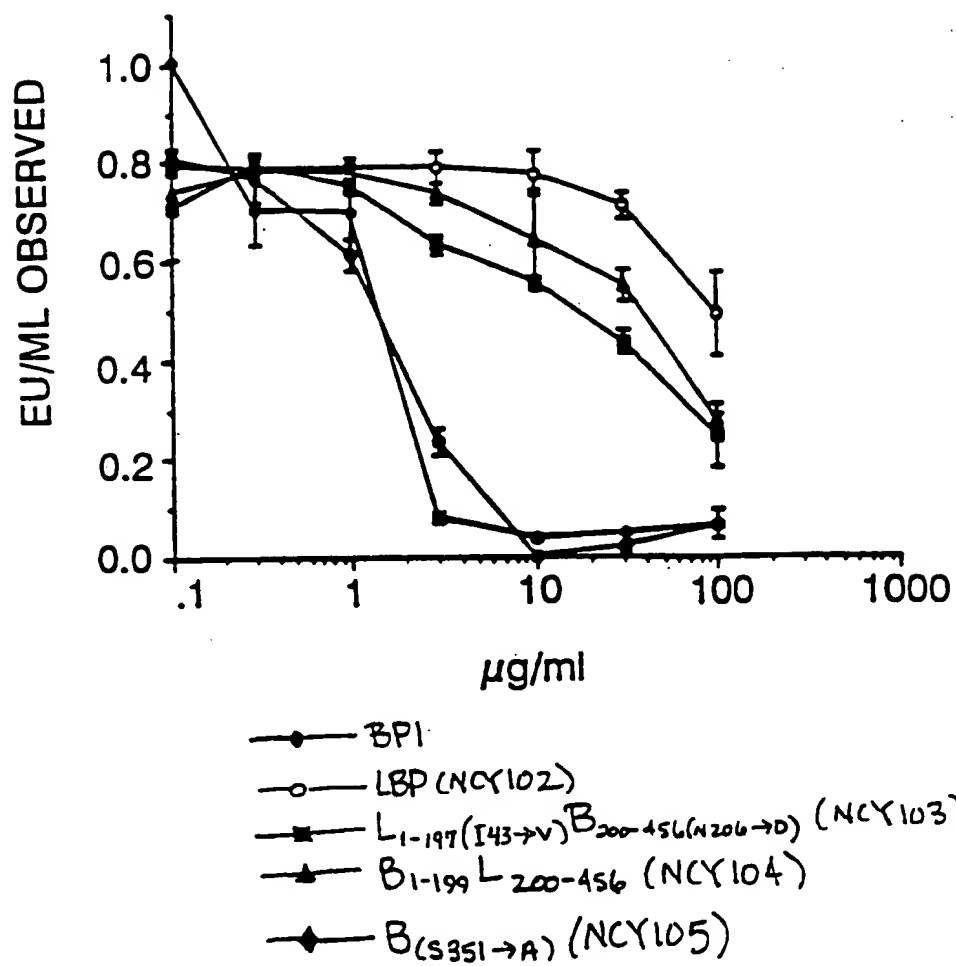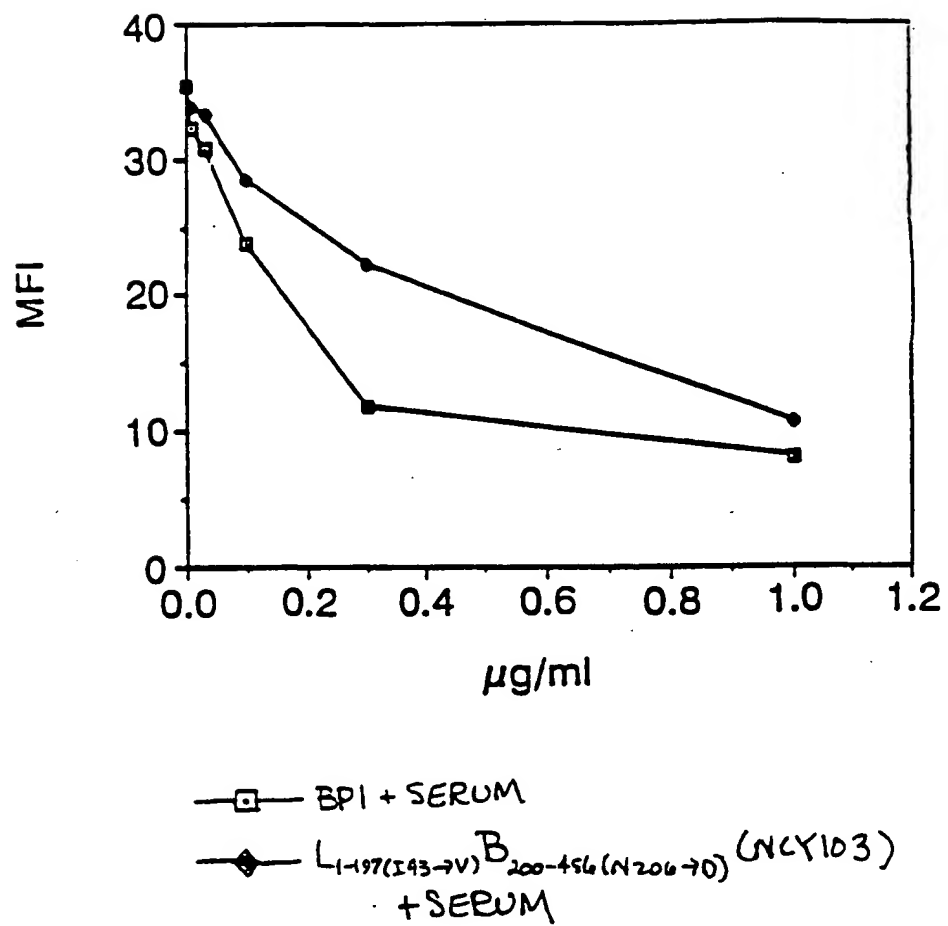
FIGURE 9

FIGURE 10

—●—BPI
—○— LBP (NCY102)
—■— L$_{1-197(I43\to V)}$B$_{200-456(N206\to D)}$ (NCY103)
—▲— B$_{1-199}$L$_{200-456}$ (NCY104)
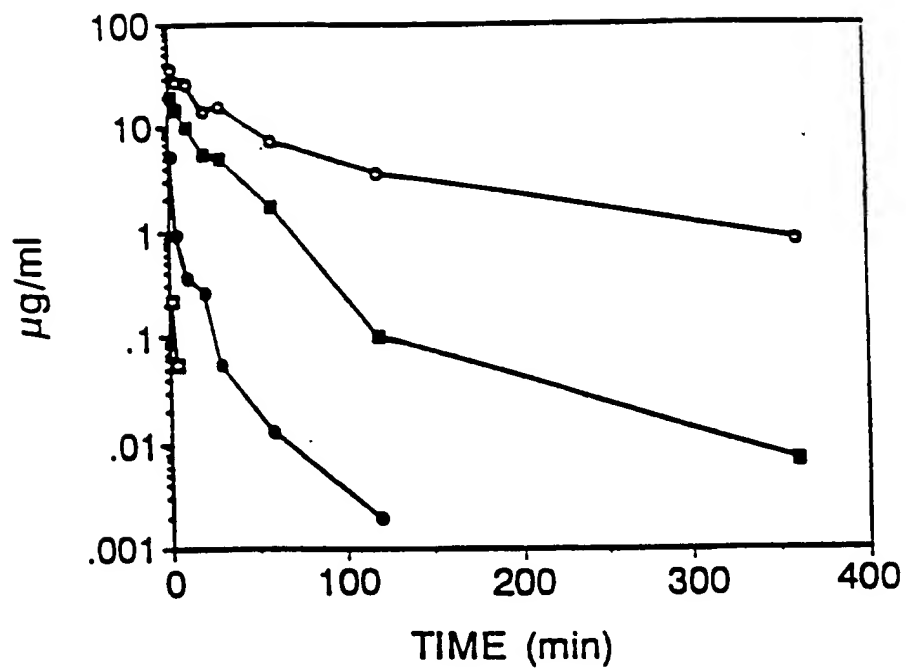
FIGURE 11

—●— BPI (5mg/kg)

—o— LBP (NCY102) (5mg/kg)

—■— L$_{1-197(I43→V)}$B$_{200-456(N206→D)}$ (NCY103) (2.5 mg/kg)

—□— B$_{1-199}$L$_{200-456}$ (NCY104) (1 mg/kg)
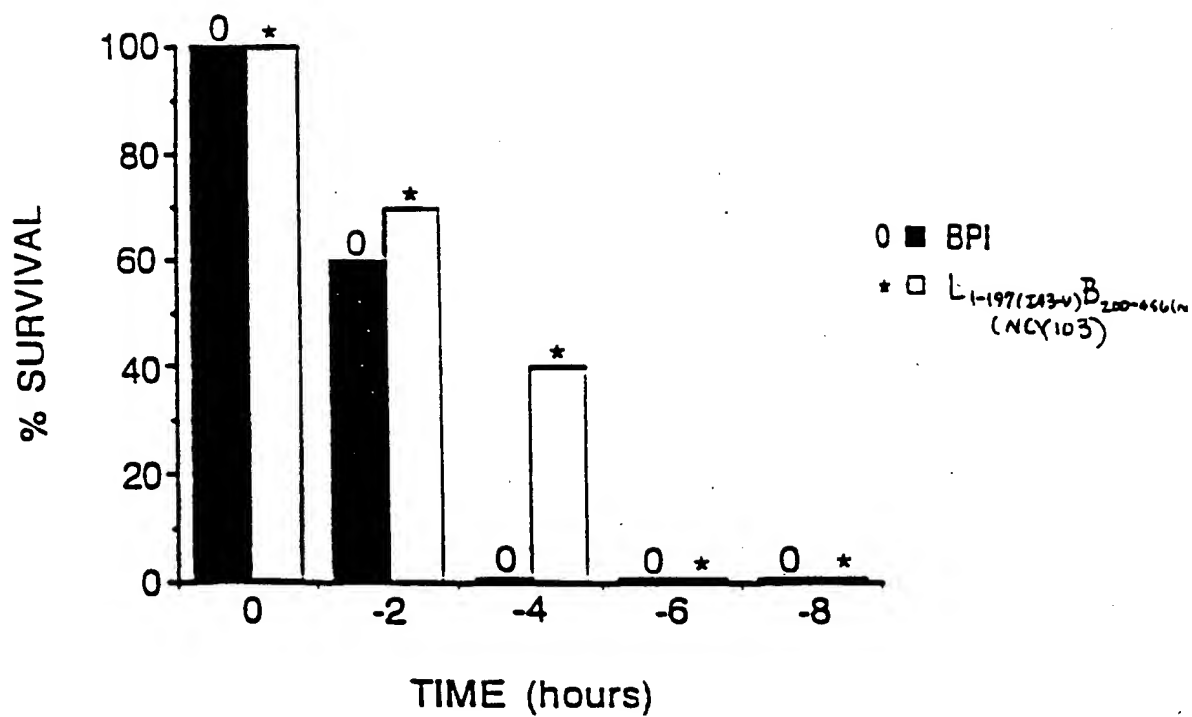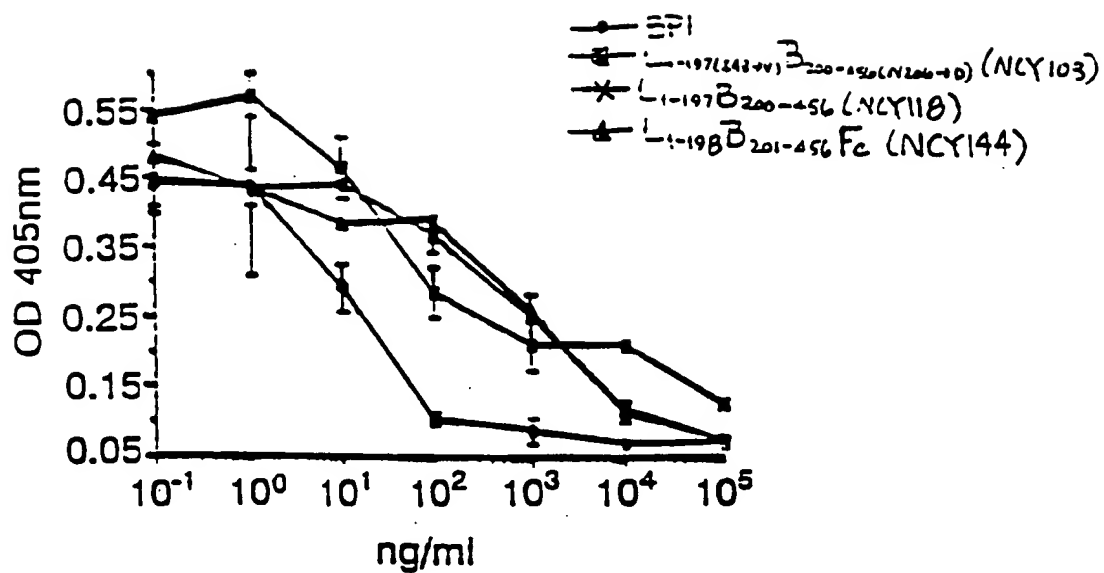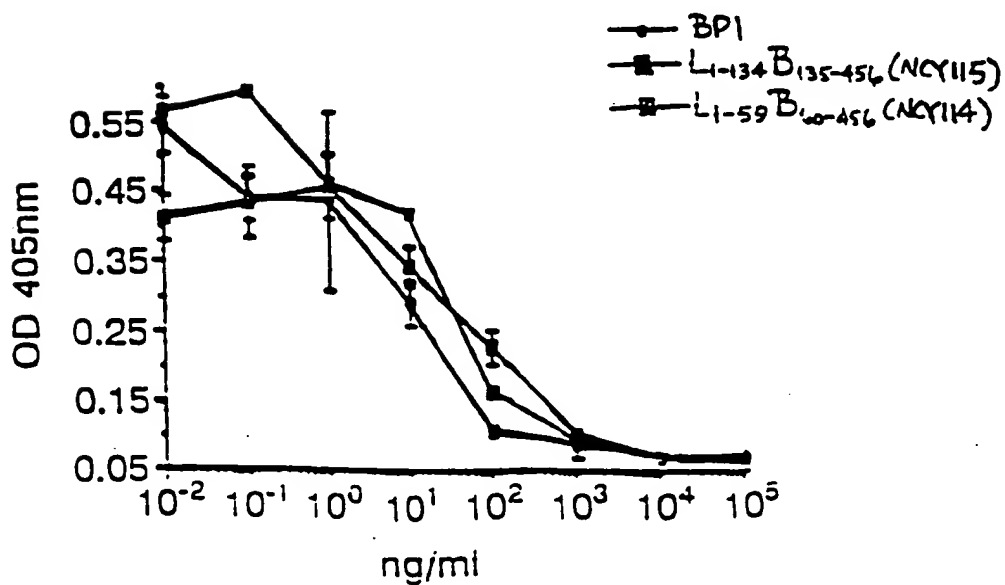
FIGURE 12

FIGURE 13A

Legend:
- BPI
- L_1-197(243→V)B_200-456(N266→D) (NCY103)
- L_1-197B_200-456 (NCY118)
- L_1-198B_201-456 Fc (NCY144)

Y-axis: OD 405nm
X-axis: ng/ml

FIGURE 13 B

Legend:
- BPI
- L_1-134B_135-456 (NCY115)
- L_1-59B_60-456 (NCY114)

Y-axis: OD 405nm
X-axis: ng/ml

FIGURE 13C

FIGURE 14A

FIGURE 14B

FIGURE 15

—o— LBP

—●— L(1-197(I13→V)) B200-456(N206→D) (NCY103)

—▲— B1-199 L200-456 (NCY104)

—□— L1-359 B360-456 (NCY117)

—■— PLL
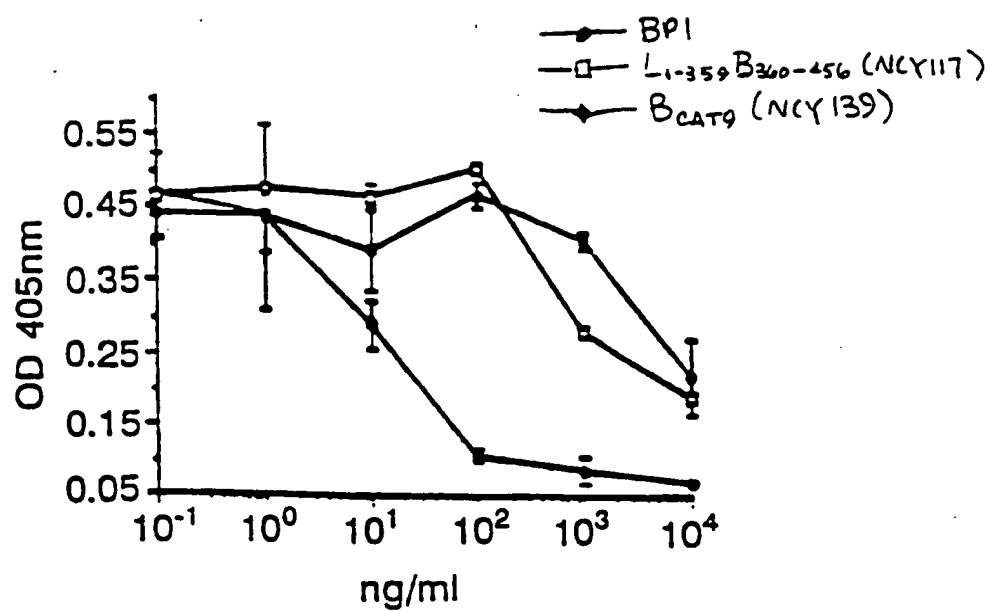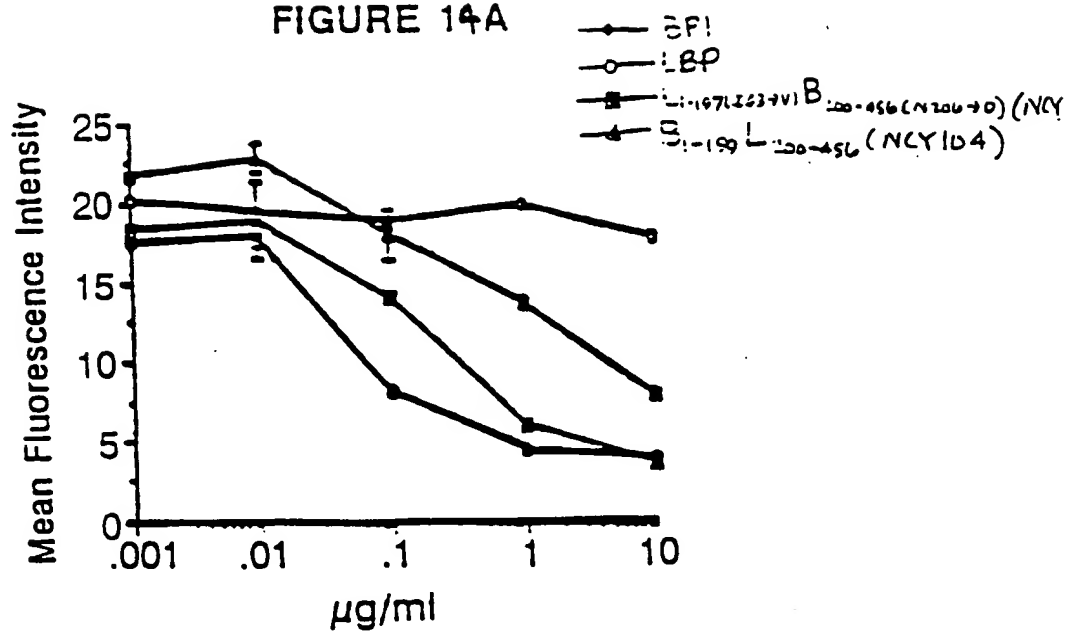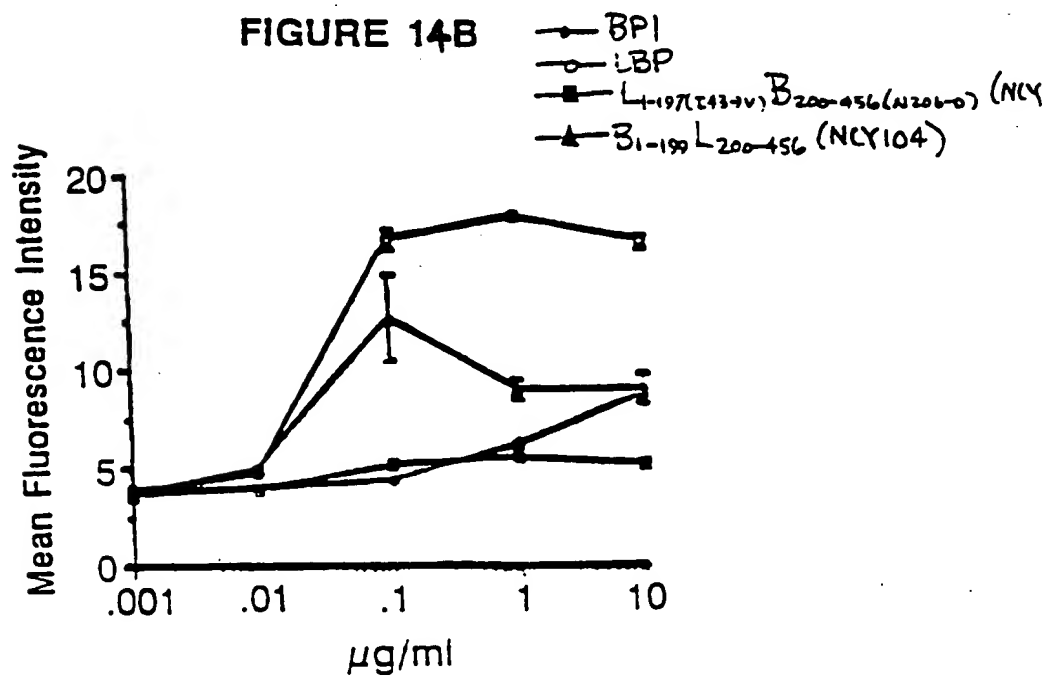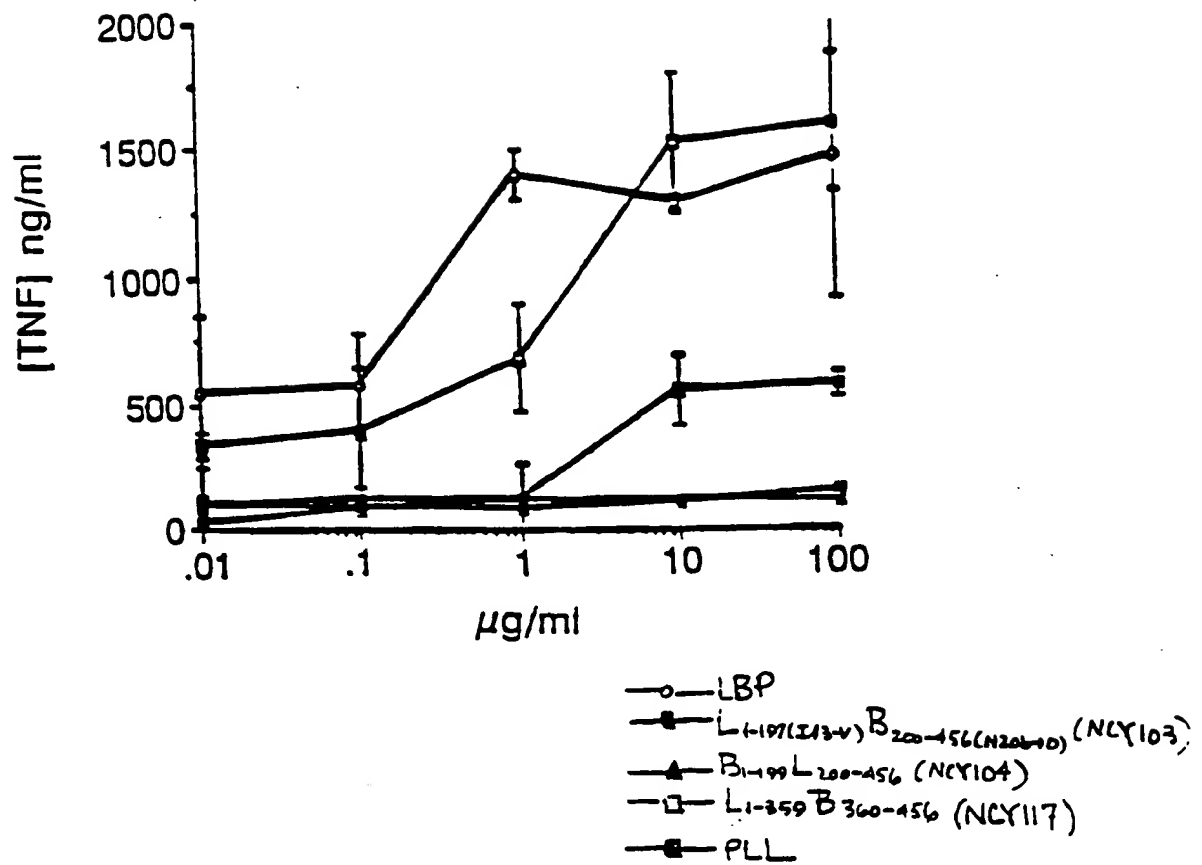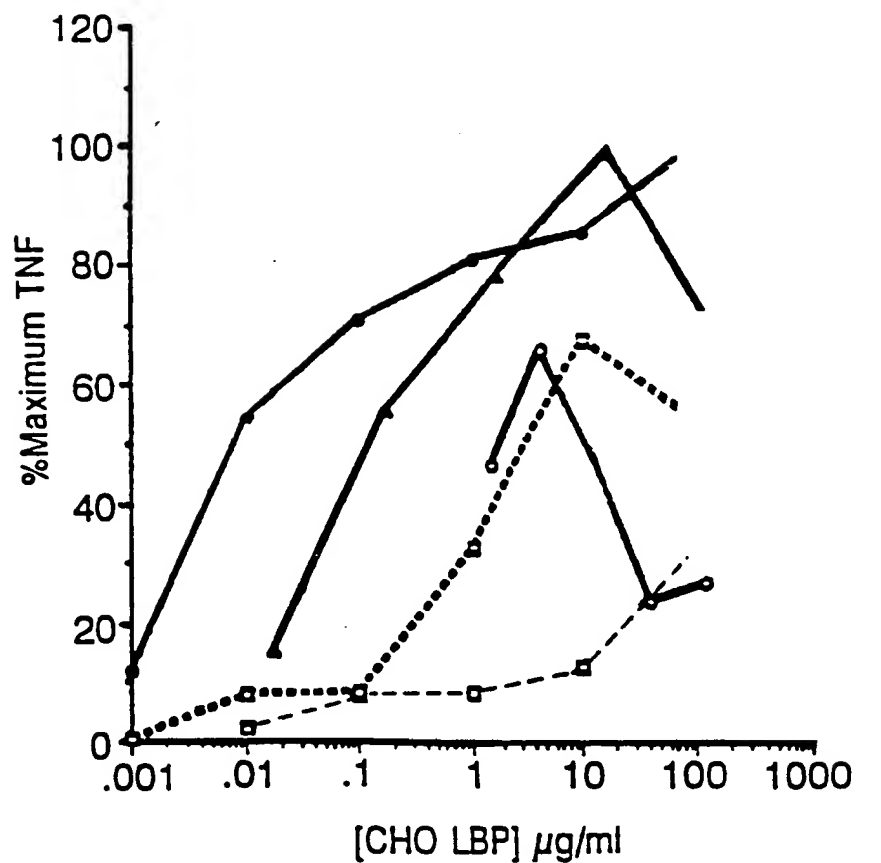
FIGURE 16

LBP (NCYlo2)(n=3)

$L_{1-198}B_{202-275}L_{274-456}$ (NCY135)(n=3)

$L_{1-134}B_{136-275}L_{274-456}$ (NCY134)(n=1)

$L_{1-275}B_{278-456}$ (NCY116)(n=3)

$L_{1-359}B_{360-456}$ (NCY117)(n=3)
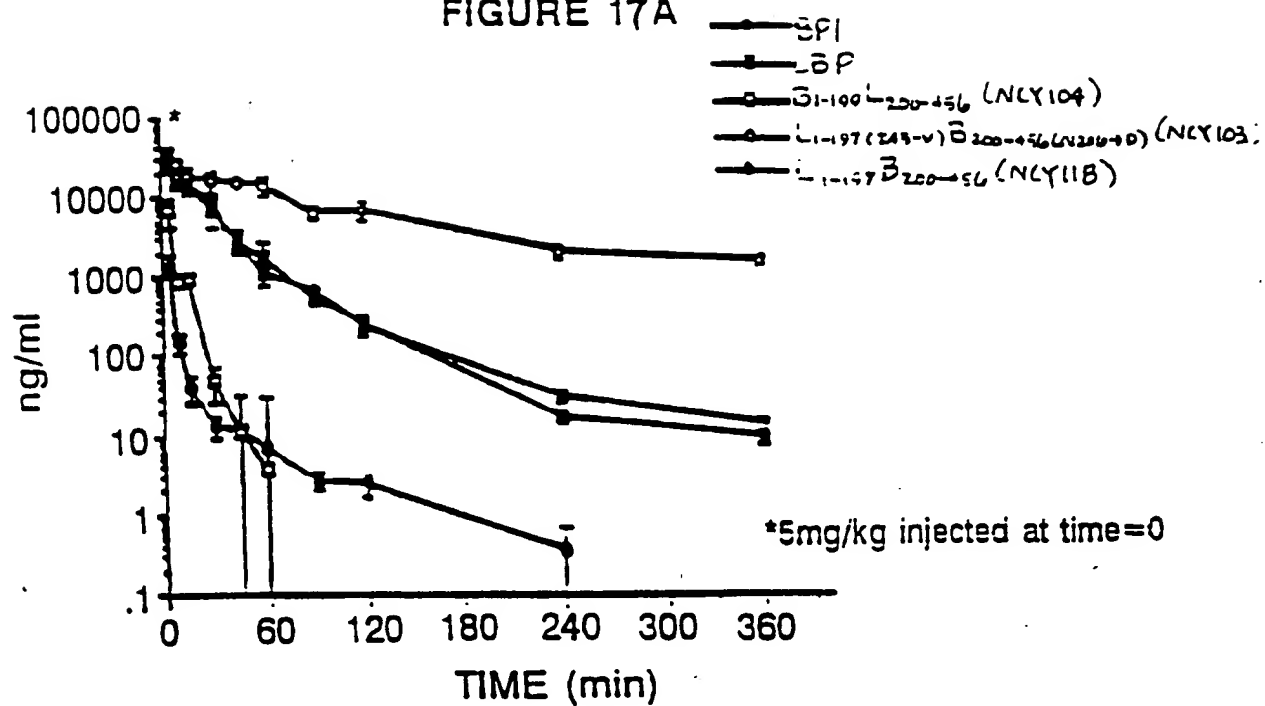
FIGURE 17A

FIGURE 17B

FIGURE 17C

*5mg/kg injected at time=0

FIGURE 17D

*5mg/kg injected at time=0
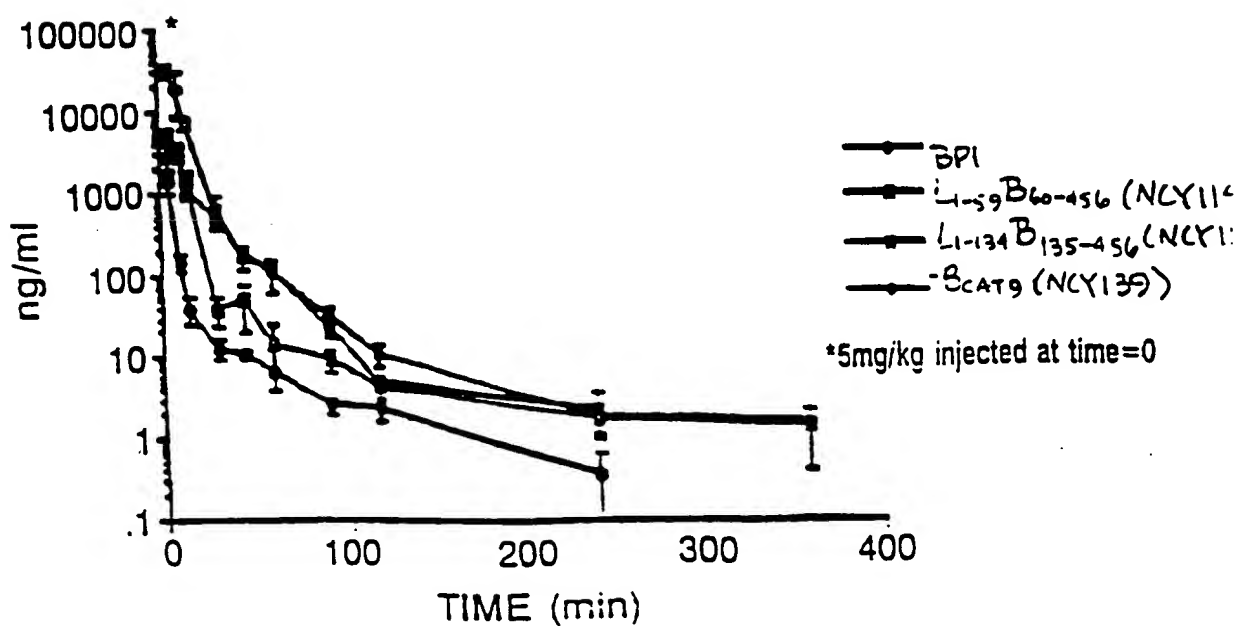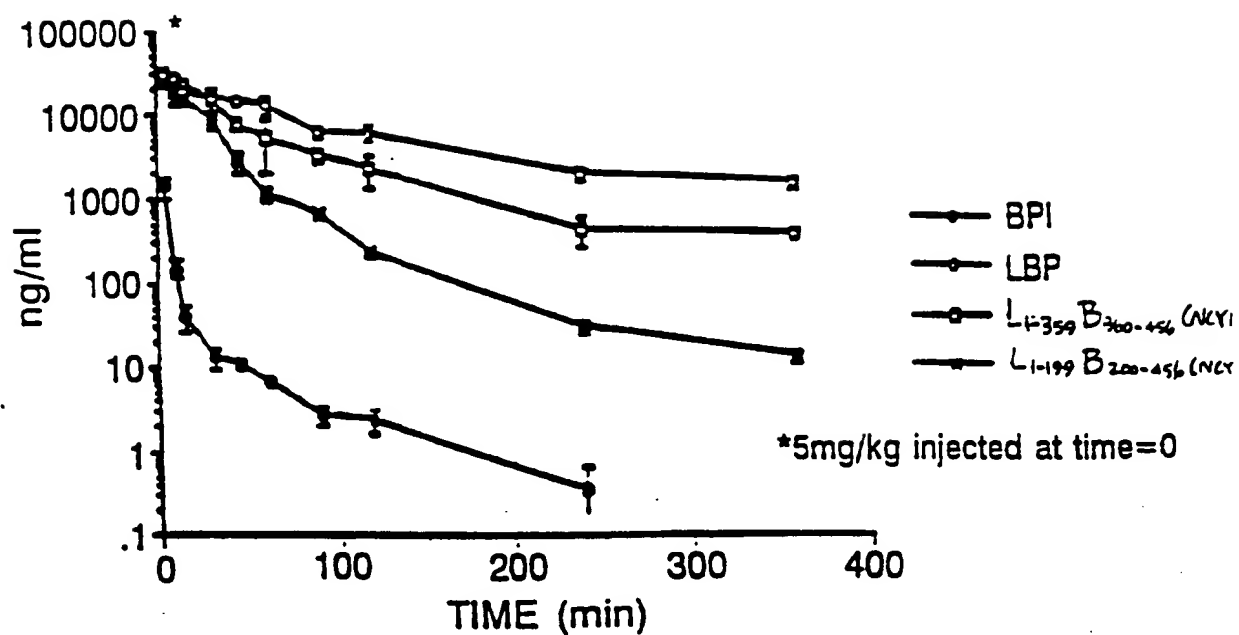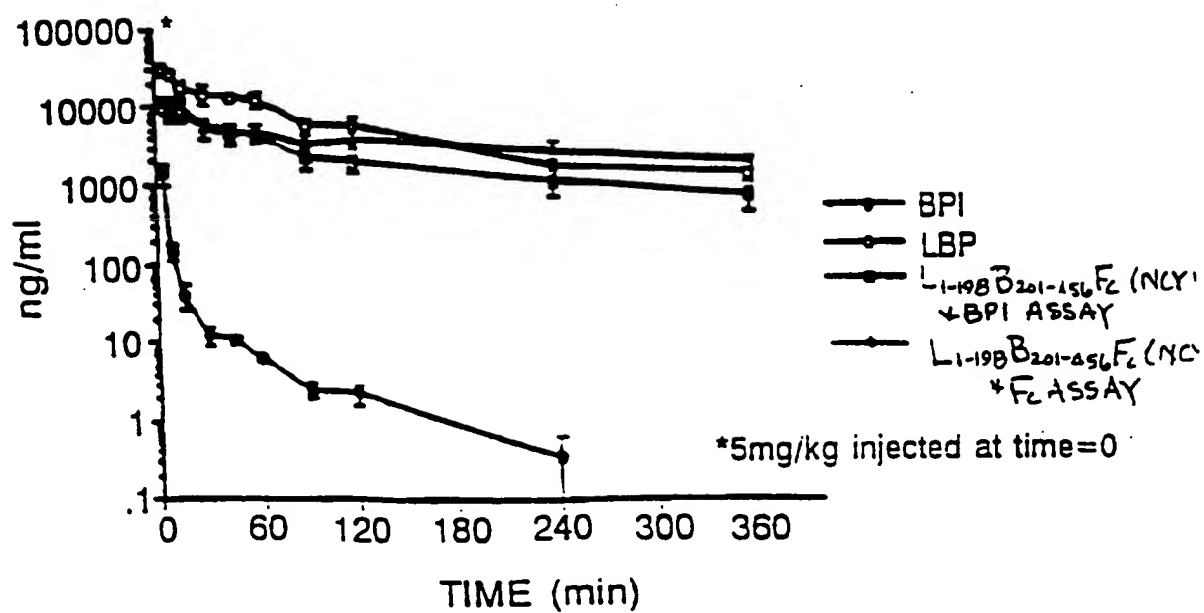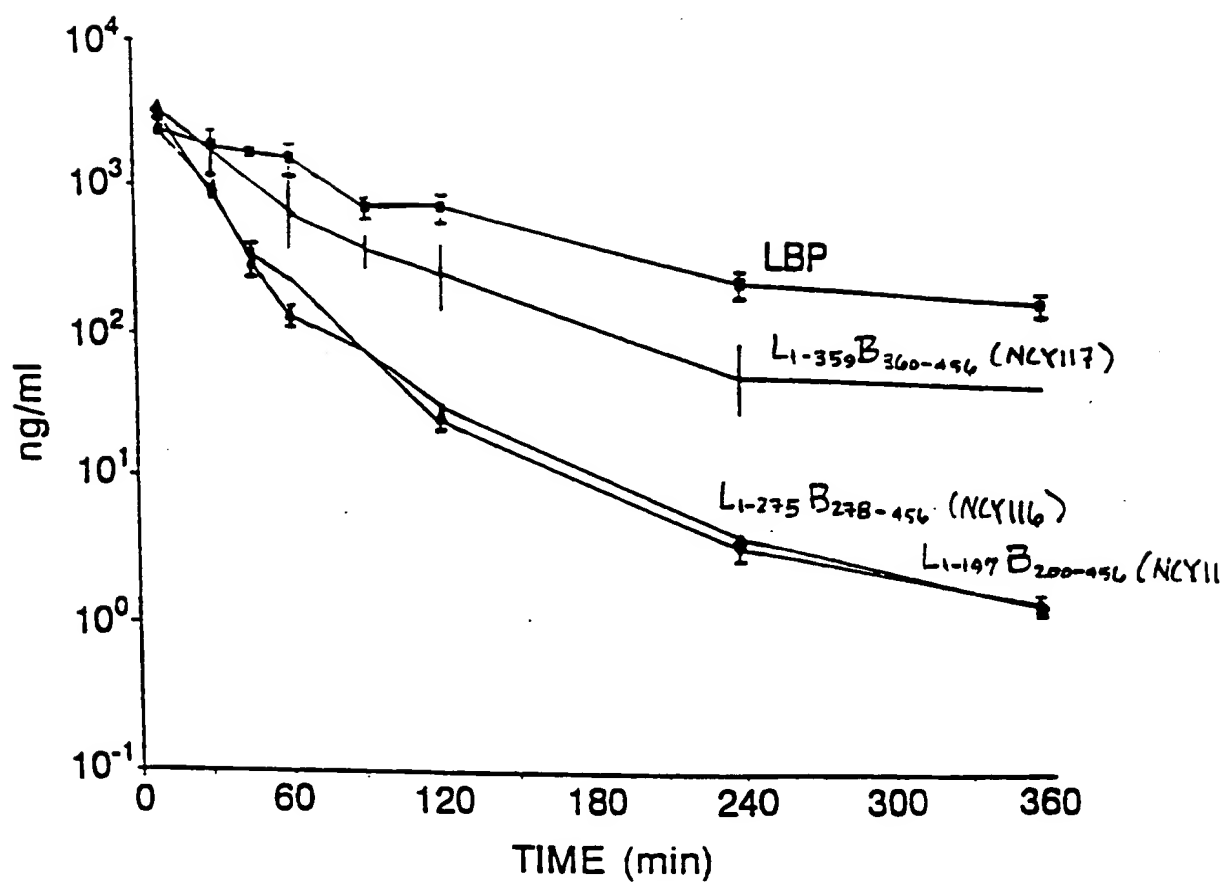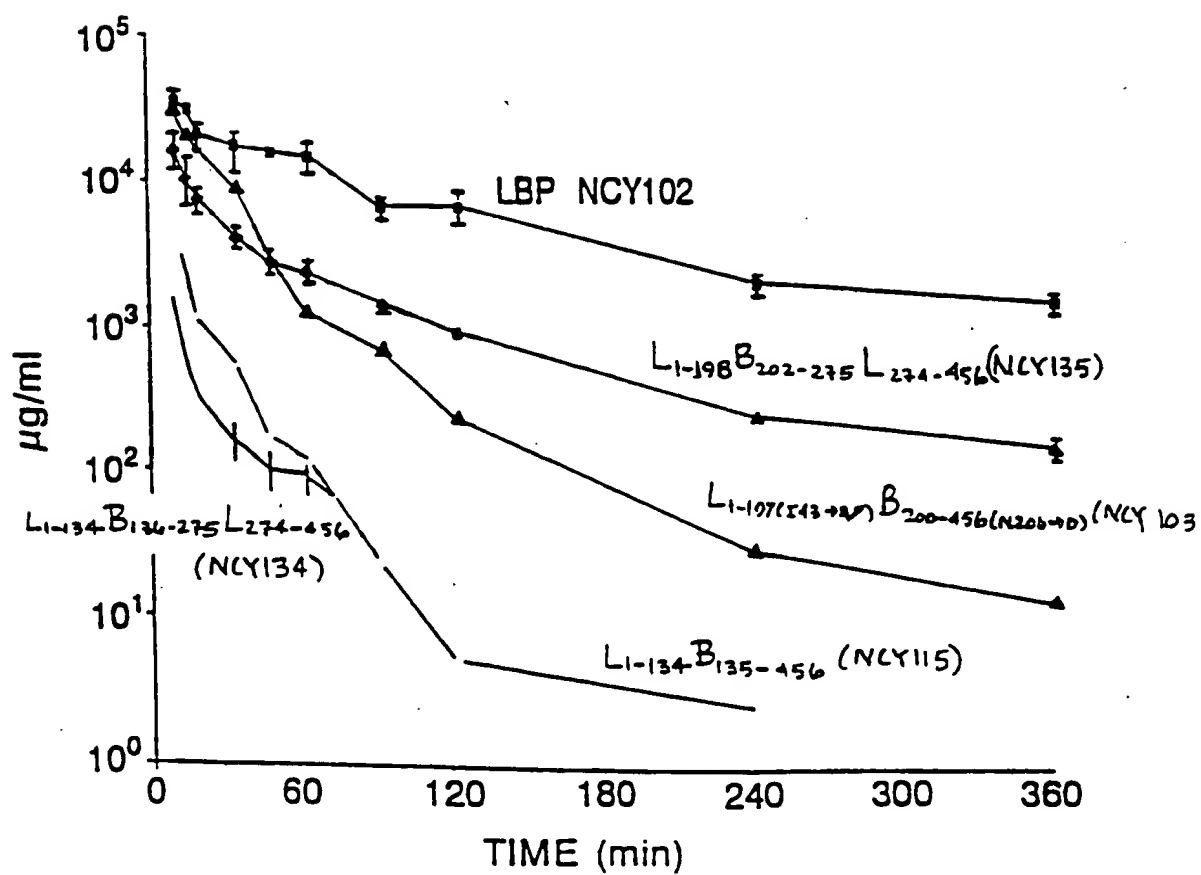
FIGURE 17E

FIGURE 17F
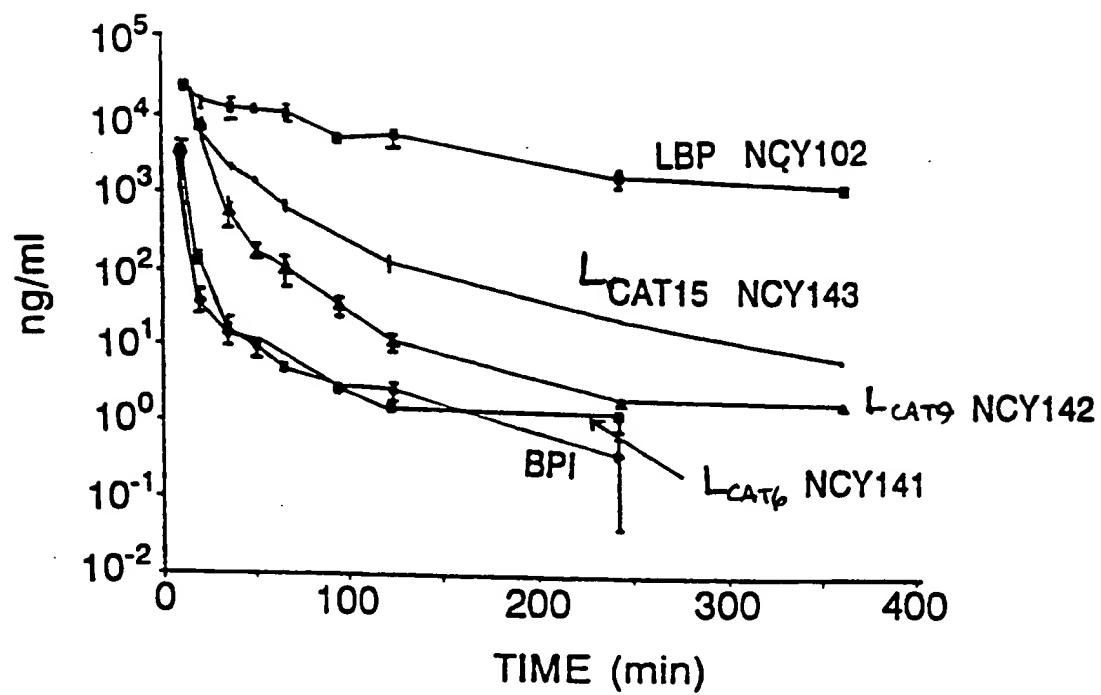
FIGURE 17G

5mg/kg compound injected i.v. at t=0
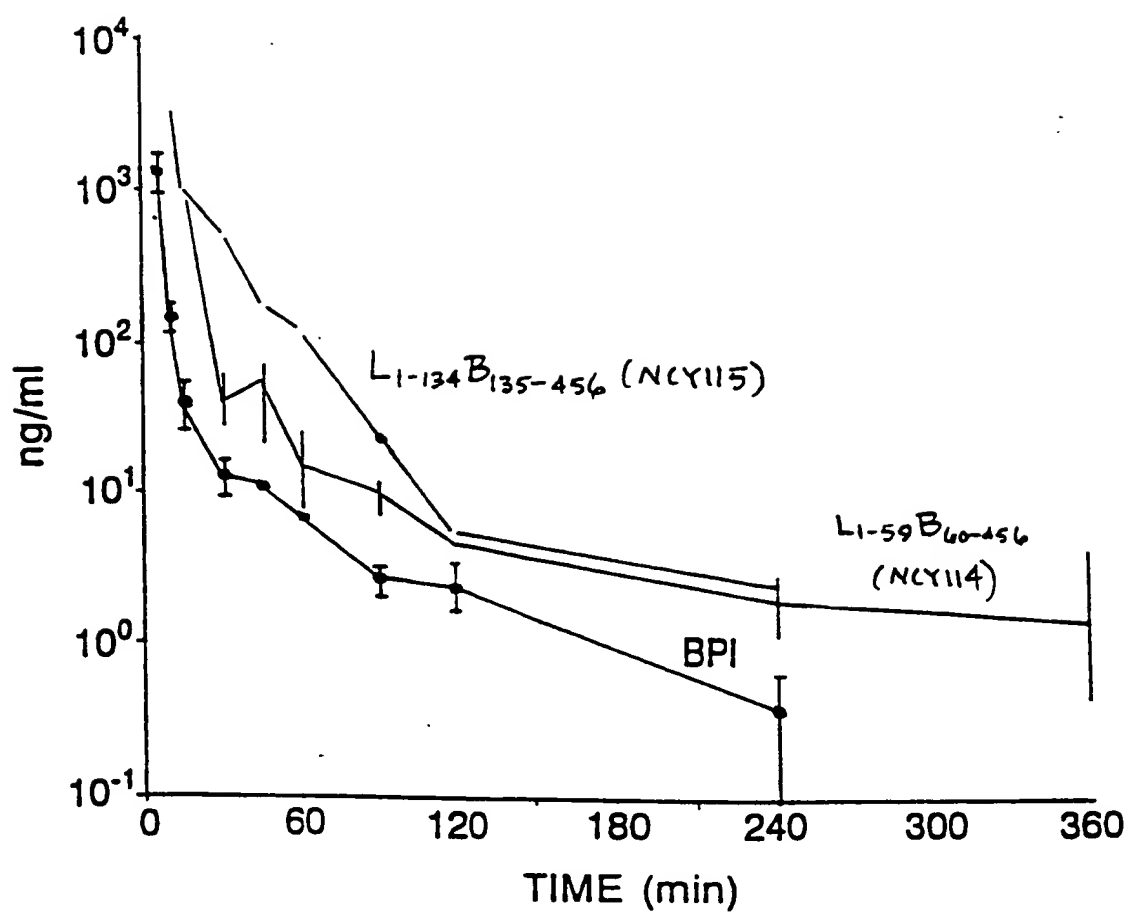
FIGURE 17H

FIGURE 18

Lane Number
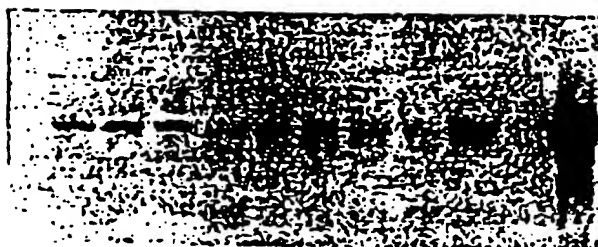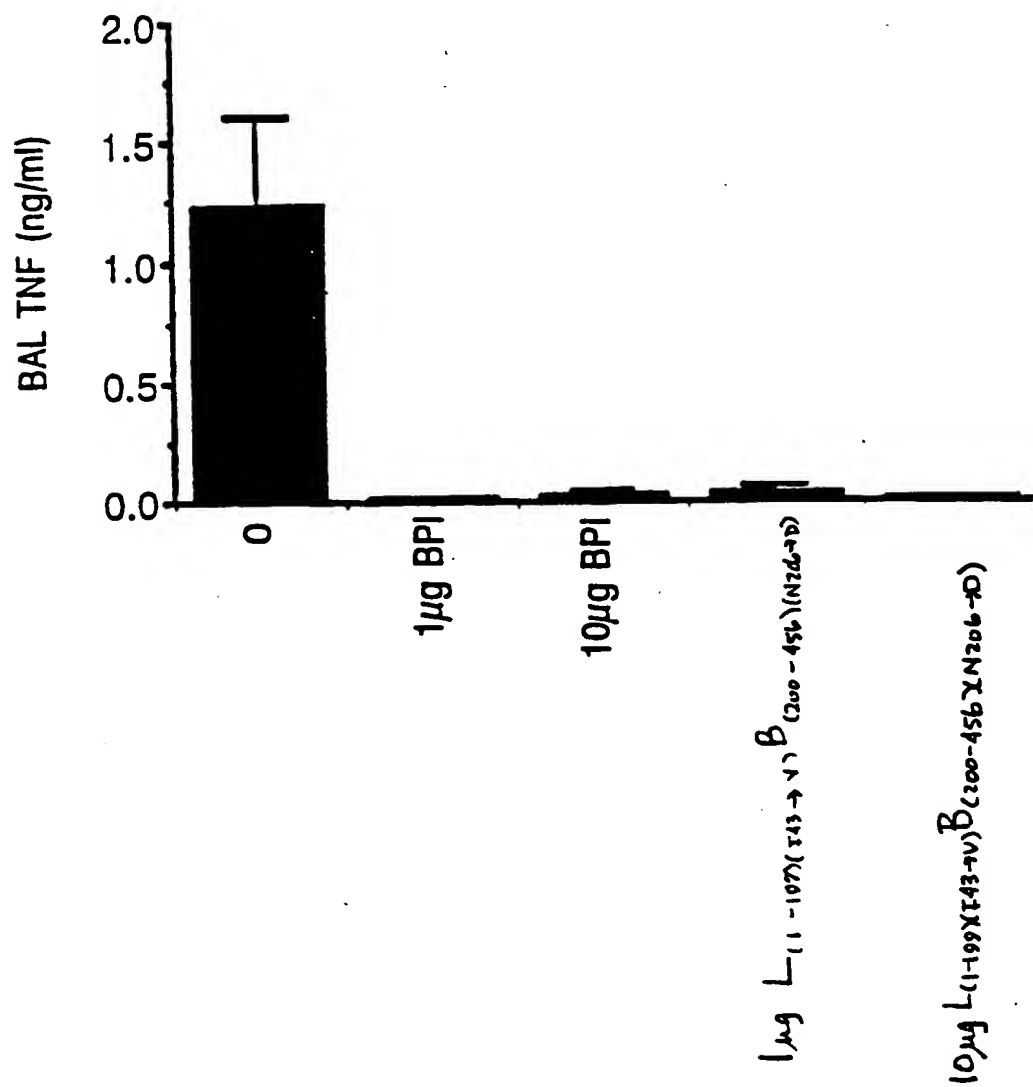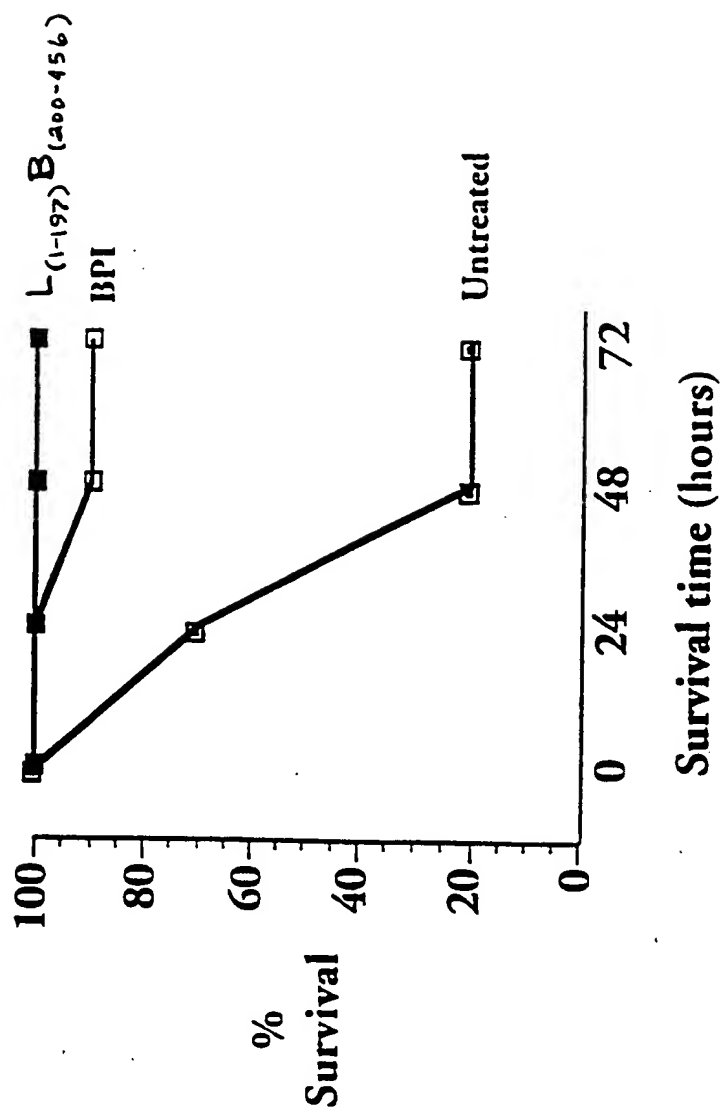
FIGURE 19

FIGURE 20

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C07H 15/12; C12P 21/06; A61K 39/00, 38/00; C07K 1/00
US CL :530/300, 350; 435/69.1, 320.1, 252.3; 536/22.1, 22.3; 514/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/300, 350; 435/69.1, 320.1, 252.3; 536/22.1, 22.3; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DIALOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,94/25476 A (SCOTT et al) 10 November 1994, see entire document.	1-38, 42
X	WO 94/18323 A (THEOFAN et al) 18 August 1994, see entire document.	14, 31, 32,
X	US 5,348,942 A (LITTLE et al) 20 September 1994, column 5, lines 40-45, column 7, lines 3-23.	14, 31, 32, 33

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 JUNE 1996	**16 JUL 1996**

Name and mailing address of the ISA/US	Authorized officer C Tsang for
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	H. F. SIDBERRY

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US96/06134

B x I Observations where certain claims were f und unsearchable (C ntinuation f item 1 f first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
   14-38, 42

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Pr test   ☐ The additional search fees were accompanied by the applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-4, 9-13, 43, drawn to method of in vivo detection of a site of Gram-negative bacterial infection, labelled endotoxin neutralizing polypeptide, composition, classified in Classes 514, subclass 2.

Group II, claim(s) 5-8, drawn to a method of in vitro detection of a Gram-negative bacterial infection, classified in Class 436, subclass 517.

Group III, claim(s) 14-38, 42 drawn to endotoxin-neutralizing polypeptides, method of making the polypeptides classified in Classes 530 and 435, subclasses 300, 350; 69.1.

Group IV, claims 39-41, drawn to DNA encoding a recombinant endotoxin binding polypeptide, vector and host cells, classified in Classes 536 and 435, subclasses 22.1, 23.1, 23.5 and 320.1 and 69.1.

Group V, claims 44, 45, drawn to methods of treatment using an endotoxin neutralizing polypeptide, classified in Class 514, subclass 2.

The inventions listed as Groups I, II, III, IV, IV do not relate to a single inventive concept under PCT Rule 13.1 because,under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is directed to a labelled endotoxin neutralizing polypeptide which is not defined. The polypeptide may be a variant of BPI or LPS in that the amino acid must not be identical to the amino acid sequences of either BPI or LPS. The methods of Group I and II are in vitro or in vivo, the method steps will differ in parameter, reagents and method steps.

The inventions of Group III are directed to polypeptides which are different from that required in Groups I and II to preform the methods of detection. The polypeptide of Group III is not the special technical feature set forth in the inventions of Group I and II. Group IV is directed to DNA which encodes a an endotoxin neutralizing polypeptide. However, variants of BPI and LPS occur spontaneously or may be achieved by chemical modifications.

Group V is directed to other methods which use the endotoxin neutralizing polypeptide, but not one which is labeled as of Groups I and II.

The special technical feature which links the inventions is known in the art, for Groups I, II and III, as SCOTT et al WO 94-25476, discloses an endotoxin neutralizing polypeptide wherein the serine at 351 has been changed to alanine. (see page 18, lines 22-27. Other variants of BPI are disclosed by SCOTT et al at page 35.

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

(a)species of BPI variants which are chimeric: claims 29-35
(b)species of BPI variants which have substitutions: 14-28, 36-38.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: the first species are directed to constructs comprising BPI or LPS and/or an immunoglobulin fragment. The second species is directed to BPI variants which have substitutions in the amino acid sequence. The variants have only substitutions. The chimeras are comprised of the LPS or the immunoglobulin fragment and thus are comprise an element not need in the variant BPI proteins.